## NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

## AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

# PATERNAL CYCLOPHOSPHAMIDE EXPOSURE EXERTS DELETERIOUS EFFECTS ON EARLY RAT EMBRYO DEVELOPMENT

by

Sara M. Kelly

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Pharmacology & Therapeutics
McGill University
Montreal, Quebec.

October, 1993.

ISBN 0-315-94644-X

Canada

KELLY, Sara M.
Department of Pharmacology & Therapeutics Ph.D.

# Paternal cyclophosphamide exposure exerts deleterious effects on early rat embryo development

## ABSTRACT

Cyclophosphamide is an alkylating agent which has deleterious effects on reproduction in both males and females. When administered to male rats in chronic low doses (6 mg/kg/day), cyclophosphamide caused a dose-dependent increase in post-implantation death of the offspring. On day 7 of gestation, two days after implantation, the inner cell mass-derived embryonic tissues were retarded or absent while trophectoderm-derived extraembryonic tissues appeared normal. The preimplantation growth of embryos sired by cyclophosphamide-treated males was affected; as early as day 3 there was a significant decrease in cell number and in DNA synthesis. On day 3, there was no ultrastructural evidence of active cell death, and embryos underwent compaction, despite their decreased cell numbers. On day 4, embryos sired by treated males had less than half the cell number of controls; this decrease was not lineage-specific. A minority of embryos sired by treated males did not cavitate and showed signs of autophagic death on day 4 of gestation. The majority of embryos sired by treated males were able to cavitate and differentiate morphologically to form small blastocysts. Thus the target of cyclophosphamide damage may be a paternal

gene more important for cell proliferation than for cell differentiation in the preimplantation rat embryo.

# Une exposition paternelle au cyclophosphamide cause des effets néfastes tôt sur le développement des embryons de rat.

## Condensé

Le cyclophosphamide est un agent alkylant causant des effets néfastes sur la reproduction autant chez le mâle que la femelle. Lorsqu'administré à faibles doses chroniques à des rats mâles (6 mg/kg/jour), le cyclophosphamide cause une augmentation des décès de la progéniture, proportionnelle à la dose employée et la durée du traitement, au niveau post-implantation. Au septième jour de la gestation, 2 jours après la nidation, le développement de la masse tissulaire embryonique interne est retardé, voir absent, alors que les cellules trophectodermiques extra-embryonnaires ne semblent pas affectées. La croissance des embryons en pré-implantation issus des mâles traités au cyclophosphamide est affectée; cela dès le troisième jour où l'on observe une diminution du nombre de cellules ainsi que de la synthèse d'ADN. Au troisième jour, il n'y a pas d'évidence de mortalité cellulaire ultrastructurale et les embryons se compactent malgré une diminution du nombre de cellules. Au quatrième jour, les embryons issus des mâles traités ont un nombre de cellules inférieur à la moitié de celui des rats témoins. Cette baisse n'est pas spécifique à la lignée cellulaire. Une minorité d'embryons issus des mâles traités n'a pas formé de blastocèle et a démontré des signes d'autophagie au quatrième jour de la gestation. Alors que

la majorité des embryons a pu former un blastocèle et se différencier morphologiquement pour former de petits blastocystes. Ainsi la cible du cyclophosphamide semble être un gène paternel ayant une plus grande influence sur la prolifération des cellules que sur la différentiation cellulaire des embryons de rat en pré-implantation.

Table of Contents

## List of Figures

Chapter IV.

## Preface

### Format of the Thesis

This thesis comprises three papers, which are included almost entirely in the form in which they were submitted for publication. Connecting texts are provided in accordance with section B(2) in the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research of McGill University, which states the following:

"Manuscripts and Authorship:
Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. Their thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to

the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary. Additional material (procedural and design data, as well as description of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

The Introduction, Chapter I, includes a general review of the background literature and the rationale for these studies. Chapter II has been published in Teratology (45: 313-318, 1992). Chapter III is published in Biology of Reproduction (50:55-64, 1994). Chapter IV will be submitted for publication.

The aspects of Chapters II, III, and IV dealing with treatment of males were carried out under the supervision of Dr. B. Robaire of the Department of Pharmacology & Therapeutics, McGill University; for this reason Dr.

Robaire's name appears as a coauthor for these papers. All experiments and measurements were performed by the candidate. The post-fixation, embedding, cutting and staining of embryos for electron microscopy were done by Ms. M. Ballak, the technician in the Electron Microscopy Laboratory in the Department of Pharmacology and Therapeutics at McGill.

Chapter V contains a general discussion of the results. The List of Original Contributions at the end of the thesis summarizes the results of Chapters II-IV.

## Acknowledgements

First, and foremost, I wish to acknowledge God, who gives me strength and inspiration, and whose creative genius is a constant source of interest and wonder to me.

I would like to thank my research supervisor, Dr. B.F. Hales, for her guidance, friendship, and encouragement throughout my studies.

I thank the Department of Pharmacology & Therapeutics for the opportunity to study in an excellent research and teaching environment.

I wish to thank Dr. B. Robaire for his guidance and critique. I also acknowledge helpful suggestions and expert advice from Dr. A. Ribeiro-da-Silva, Dr. G. Almazan, Dr. E. Daniels, Dr. L. Hermo, and Dr. J. Brawer.

I wish to thank Dr. B. Chen for letting me be an "accidental occidental" friend and for making my graduate studies so much fun. I thank Dr. C. Huang for her friendship, helpfulness, and wise advice. I thank Mr. S. Moallem for his willingness to explain the network as many times as it takes. Mr. T. Ozolins I thank for not saying "I told you so..." and for helping me defend Toronto, even if he's not a "Scarb".

I also thank Dr. C. Kapron-Brás, Ms. C. Tseng, Ms. K. Crosman, Mr. D. Lehnert, Ms. J. Pelletier, Ms. J. Qiu, Mr. R. Viger, Dr. D. Cyr, and Dr. J. Kaur, my other co-workers

for providing a friendly and cooperative research environment.

I am grateful for excellent technical assistance and advice for the electron microscopy from Ms. Marie Ballak. I also thank Mr. A. Forster for his photographic work.

I would like to thank the FCAR Centre for the Study of Reproduction and the McGill Faculty of Medicine for support during these studies. Funds for these studies were provided by the Medical Research Council of Canada.

I am grateful for the constant encouragement and support of my parents throughout my education, and for the generous hospitality of the Austin family while I have been in Montréal.

My deepest thanks go to Peter, who reminds me of what is truly important, and who has been stalwart in patience, encouragement, and love.

# Chapter I

## Introduction

Birth defects and pregnancy loss can sometimes be traced to environmental, chemical, or drug exposures. The generation of malformations, or teratogenesis, is usually associated with exposure of the conceptus to environmental or chemical agents *in utero*, through the mother. There is, however, a considerable body of experimental evidence pointing to teratogenic and/or embryotoxic effects when the father is exposed to dangerous agents before fertilization.

Because of the equal genetic contribution from both maternal and paternal germ cells to the zygotic genome, genetic defects in the germ cell of either parent can be of equal consequence. Mutations in genes inherited from the father can interfere with embryo development. The examination of male-mediated effects on development will lead to an increased understanding of the paternal contribution in development, and to the prevention of avoidable fetal loss and/or birth defects in humans.

This introduction is divided into three sections. The first section outlines the current human and animal evidence for paternally-mediated effects on development, including the model used in this thesis. The second section provides a brief description of normal and abnormal preimplantation embryo development. The final section provides the rationale and formulation of the experiments.

A. Paternally mediated developmental toxicity

1. Human Evidence

Exposures of men to dangerous agents are primarily of two types: environmental and medical.

Environmental exposures can include those in the occupational as well as the home environment. A number of studies have shown a link between abnormal progeny outcome and environmental and/or occupational exposures of men (reviewed in Hales & Robaire, '93). Paternal occupation as a motor vehicle mechanic (McDonald et al. '89), painter (Brender et al. '90), fireman (Olshan et al. '90), janitor, forestry worker, printer, plywood mill worker (Olshan et al. '91), art worker or textile worker (Savitz et al. '89) is associated with increased risk of undesirable progeny outcome. Clearly, men with these and similar occupations need to be aware of the risks of occupational exposures prior to conceiving a child.

Medical exposures of men can include therapeutic radiation and therapeutic drug exposures. Irradiation can cause transient or longer-term azoospermia in men depending on dose (Rowley et al., '74). Attempts have been made to link environmental radiation exposure from a nuclear power plant in England with cancer in progeny, but reports have been contradictory and the link remains controversial (Gardner et al '90; Baverstock '91; Kinlen '93).

The proportion of malformed children fathered by men

who have undergone chemotherapy does not seem to differ from the control group or the general population (Senturia et al '85; Fried et al. '87). However, treatment of men with anticancer drugs often results in transient or permanent infertility (Byrne et al., '87).

An insufficient number of patients have been studied to detect a relatively low increased risk with paternal anticancer drug exposure. Treatment of men with the anticancer drug cyclophosphamide can have effects on germ cell numbers, resulting in oligozoo- and azoospermia, with recovered fertility in many but not all cases (Fairley et al., '72; Qureshi et al., '72; Fukutani et al., '81; Charak et al., '90).

In cases where sperm numbers are sufficient, what is diagnosed as infertility may indicate failure in very early development. Such failure can lead to spontaneous abortion prior to or just after implantation, which occurs approximately one week after conception in humans. Women are usually unaware of their pregnancy until two weeks after conception, and thus would not consider such an early failure in development as a pregnancy loss. Because of this, defects in the germ cells which result in early embryo loss may be misinterpreted as impaired fertilization.

Because of the limitations of human studies, researchers have turned to animal models to study male-mediated effects on progeny outcome.

2. Animal Evidence

There is an extensive collection of animal studies providing experimental evidence for a variety of paternal effects on progeny outcome.

The fertility of the male can be affected by paternal exposures which decrease the number of spermatozoa produced so that there are not enough sperm present to reach the ovum, or which alter the ability of the sperm to penetrate the ovum once it is reached. Some paternal exposures cause the failure of the embryo at a point in its development, leading to embryo death either before or after the embryo implants in the uterine epithelium. Thus, depending on the stage of development, developmental toxicity is manifested as infertility, preimplantation loss or postimplantation loss.

Malformations are caused when failures during development are not lethal to the embryo but alter processes of organ formation (Wilson, '77). Offspring are born alive but with observable defects, which can be structural, such as limb malformations (Kochhar, '73), or behavioral, such as decreased learning capacity (Auroux et al., '86). Often a single litter will demonstrate a spectrum of effects, and even those offspring which appear to be normal can carry subtle genetic defects which are expressed in the next generation (Hales et al., '92).

A number of agents affect progeny outcome through a

paternal exposure (Robaire et al., '85). X-irradiation (Goldstein & Spindle, '76; Kirk and Lyon, '84), methyl methanesulfonate (Ehling et al., '68; Brewen et al., '75), N,N'-methylenebisacrylamide (Rutledge et al., '90), and cyclophosphamide (Fritz et al., '73; Moreland et al., '81; Trasler et al., '85,'86,'87) are all examples of agents which have paternally-mediated dominant lethal effects. By some mechanism these agents are able to act through the father to affect the development of the embryo in the mother.

3. Mechanisms

There are a number of mechanisms by which paternal drug exposure could result in adverse effects on development.

*a. Direct effect on the zygote*

One possible route of drug action is a direct effect on the ovum when the drug is transferred to the female in the semen. Indeed, cyclophosphamide can act via seminal transmission to cause a significant increase in preimplantation loss (Hales et al., '86). Alternatively, it has been proposed that damage to the outer surface of the spermatozoon could be delivered to the ovum at the fusion of membranes following penetration and have effects on embryo development (Wiley, '90).

*b. Indirect effect on sperm via the male reproductive tract*

Another mechanism by which paternal drug exposure can result in defective embryonic development is by secondary effects of drug action on the male reproductive tract. Alterations in hormone levels and/or reproductive organ function could result in changes in the spermatozoa. For example, vinyl chloride acts to cause both preimplantation and postimplantation embryo loss by causing inflammation of the epididymis, which affects the maturation of the spermatozoa (Chellman et al '86). Similarly, removal of the male accessory sex gland secretions can affect the rate of preimplantation development in the golden hamster (O et al., '88). In this case the sperm, the ovum, or both could be affected by this change in semen composition.

*c. Direct effect on the sperm*

The final and most obvious mechanism of drug action is a direct action on the male germ cells. Spermatogenesis is a precisely coordinated process taking 51.6 days in the Sprague-Dawley rat (Clermont, '72). The male stem cells, or spermatogonia, undergo four mitotic divisions over a 2-2.5 week period before undergoing a fifth division to form spermatocytes. The spermatocytes develop over another 2-2.5 week period, then undergo meiosis to form spermatids. Spermiogenesis, the process of transformation of spermatids into spermatozoa, occurs over a period of approximately 3 weeks. The spermatozoa pass from the testis through the efferent ducts into the epididymis where they acquire their

fertilizing potential (Orgebin-Crist, '69). The spermatozoa then take between 10-14 days to pass through the epididymis (Robaire & Hermo, '88).

The precise timing of spermatogenesis in the rat allows for identification of germ cell stages vulnerable to drugs. The effects of drug exposure on progeny outcome can be analyzed by mating males at varying intervals after a single dose treatment, then correlating any effects on the progeny to the germ cell stage at which the fertilizing spermatozoa were exposed. For example, effects on progeny outcome within the first two weeks after treatment would correlate to an effect on epididymal spermatozoa. Similarly, effects seen only 3 to 5 weeks after exposure would indicate that germ cells first affected were spermatids. The effects of chronic exposures to drugs can also be assessed in this manner, to determine if the effects are dependent on the stage at which the germ cell was first exposed.

Many of the drugs which affect embryonic development through a paternal mechanism are alkylating agents. It has been known for some time that the effects of various alkylating agents on the fertility of the male can be stage-specific (Jackson '64; Meistrich et al., '82). Both DNA and protein are targets for alkylation in the sperm. Because no repair takes place in postmeiotic germ cells (Sega, '74), they may be particularly sensitive to DNA damage. There have been a number of studies of DNA damage to germ cells evoked

by treatment of males with various anticancer drugs (Sotomayor & Cumming '75; Lyon, '81; Abraham & Fränz '83; Matsuda et al., '89).

4. Model System - Cyclophosphamide

Cyclophosphamide has been used extensively as a model teratogen. Cyclophosphamide is activated metabolically to two cytotoxic compounds, phosphoramide mustard and acrolein, both of which contribute to its teratogenicity (Hales, '82). It has been shown to be carcinogenic (Schmahl & Habs, '79) and mutagenic (Hales, '82), and is able to alkylate DNA, primarily on the N-7 group of guanine (Benson et al., '88). Cyclophosphamide is used as a model drug for paternally-mediated effects on development. It has been clearly shown in rats that cyclophosphamide dramatically affects the development of offspring without a major effect on the fertility of the male (Trasler et al., '85, '86, '87, '88; Trasler & Robaire, '88). Chronic treatment with low doses of cyclophosphamide caused fetal malformations and as much as 95% embryolethality (Trasler et al., '85). Those offspring which were apparently normal carried heritable genetic defects, which led in subsequent generations to malformations (Hales et al., '92) and behavioral defects (Adams et al., '81. '82; Auroux et al., '86,'88; Dulioust et al., '89; Fabricant et al., '83; Velez de la Calle et al., '89). The same treatment did not alter serum hormones in the male and led to only transient decreases in the numbers

of condensed spermatids and spermatozoa in the testis and epididymis and decreases in weights of the epididymis, ventral prostate and seminal vesicles (Trasler et al., '87). The very subtle morphological and biochemical changes in the epididymal epithelium and increase in the incidence of sperm bearing abnormal midpieces (which are likely to be incapable of fertilization) did not explain the dramatic effects on embryogenesis (Trasler et al., '88; Trasler & Robaire, '88).

The effects of paternal cyclophosphamide on progeny outcome were not only dose-related but also dependent on the period of treatment. The most dramatic effect produced by a dose of 5.1 mg/kg/day of cyclophosphamide was an increase in post-implantation loss, which reached a plateau level of 80% when the male was exposed for 4 weeks. This pattern of increase to a maximal effect after four weeks of exposure would seem to indicate some stage-specificity of germ cell damage by cyclophosphamide. The spermatozoa which fertilized the embryos were first exposed to cyclophosphamide at the spermatid stage four weeks earlier. That this dramatic postimplantation loss was reversible by 4 weeks after cessation of treatment (Hales & Robaire, '90) is further evidence of a spermatid-specific effect. This postimplantation loss after 4 weeks of treatment consisted of peri-implantation or early postimplantation resorptions. Increased preimplantation loss was evident only after 5-6 weeks of treatment, suggesting an effect of cyclophosphamide

on germ cells first exposed as spermatocytes and early spermatids. An increase in malformed and growth retarded fetuses was seen after 7-9 weeks of treatment, reflecting an effect of paternal cyclophosphamide on germ cells first exposed to the drug as spermatogonia.

After fertilization the highly condensed DNA forming the nucleus of the spermatozoon decondenses to form the paternal pronucleus. Chronic treatment with 6 mg/kg/day cyclophosphamide for six weeks is able to change the timing of sperm decondensation in vitro (Qiu et al., manuscript in preparation); this may be due to the ability of cyclophosphamide to cross-link DNA or protein.

The long-term effects of paternal cyclophosphamide exposure such as postimplantation loss, malformations, and heritable defects likely arise from permanent damage to the paternal genome. Cyclophosphamide induces a variety of damages in the male germ cell, including chromosome aberrations (Rathenberg & Müller, '72; Pacchierotti et al., '83; Jenkinson & Anderson, '90), translocations (van Buul, '84), synaptonemal complex damage (Backer et al., '88), and micronucleus formation (Lähdetie, '88) in the DNA of male germ cells. The mechanism of cyclophosphamide action is presently unclear. The normal processes of embryogenesis may be altered by the misexpression of paternal genes sustaining cyclophosphamide-induced damage or by the formation of mutant proteins.

B. Embryogenesis

1. Normal preimplantation development in the rat

At fertilization, the sperm nucleus provides the haploid male pronucleus, which combines with the haploid maternal pronucleus to form the diploid embryonic genome. The embryo divides several times to form a morula, a cluster of approximately 14-22 cells, in the middle of the third day of gestation (Pampfer et al., '90). The morula contains inner and outer cells (Dvorak '78) which differentiate into two cell lineages, the inner cell mass and the trophectoderm cells (Pedersen, '86). By the fourth gestational day the embryo has become a blastocyst in which these two cell lineages are clearly apparent (Pampfer et al., '90). The blastocyst is a sphere of trophectoderm cells enclosing the inner cell mass at one pole and a cavity of fluid called the blastocoele. The blastocyst implants into the uterus on the fifth gestational day (Pampfer et al., '90). The inner cell mass cells give rise to the fetus and its membranes, whereas the trophectoderm cells give rise only to extraembryonic placental tissues (Gardner & Rossant, '76; Rossant et al., '83).

Recently the expression of paternal (and therefore embryonic) genes has been detected in embryos as early as the one-cell stage (Sprinks et al., '93). The production of proteins is gradually taken over by the embryonic genome as the original maternally-transcribed mRNAs which were present

in the ovum are degraded (Telford et al., '90; Schultz & Heyner, '92).

There is clear evidence that the expression of certain genes is different depending upon their inheritance. For example, only the copy of the insulin-like growth factor II (IGF-II) gene inherited from the father is expressed during embryo development (DeChiara et al., '91). Even if the paternal copy is absent, there is no compensatory expression of the maternal allele. Parent-specific expression of genes is reversible over generations. The IGF-II gene which is not expressed when inherited from the mother is expressed in the next generation if it is inherited from the father. The same "imprinting" phenomenon exists in the opposite manner for some genes such as the receptor for IGF-II where only the maternal copy of the gene is expressed (Barlow et al., '91). Thus during germ cell maturation some epigenetic alteration changes the genome such that certain genes are turned on or off in a parent-specific manner.

2. Abnormal Preimplantation Development

There are a number of ways in which preimplantation development can be disrupted. A deletion or modification of genetic material could interfere with development by altering the normal sequence of gene expression. Damage to embryonic cells due to an external agent, be it medical or environmental, could also seriously interfere with

development.

Nuclear transfer experiments showed that diploid mouse embryos lacking a maternal or paternal genome were unable to complete their development (McGrath & Solter, '84, Barton et al., '84; '85; Surani et al., '84). These experiments also showed that those embryos lacking a maternal genome were particularly poor in embryonic tissue development, whereas those embryos lacking a paternal genome showed poor extraembryonic tissue development (Surani '86). This would be consistent with a requirement for imprinted genes from both parents. Such results would also suggest that paternal genes are important in the development of the extraembryonic tissues, and that damage to the paternal genome may result in poor extraembryonic development.

A number of inherited mutations can cause abnormal preimplantation development, usually by cessation of division (Magnuson, '86; Magnuson & Epstein, '87). Some examples of these include mutations in the *t* complex (Wudl et al., '77), the oligosyndactyly mutation (*Os/Os*; Magnuson & Epstein, '84), and lethal yellow ($A^{Y}/A^{Y}$; Pedersen, '74). Another mutation, the $c^{25H}$ deletion, is one of the earliest acting genetic abnormalities, wherein the homozygous embryos cease dividing between the 2- and 6-cell stage, and die one or two days later (Lewis, '78; Nadijcka et al., '79).

Although external agents are usually examined for teratologic effects during the organogenesis stage, many of

them can affect preimplantation development as well. Exposure of zygotes to neutrons or X-rays caused fetal loss and malformations (Pampfer & Streffer, '88). Zygotic exposure to either ethyl methanesulfonate or ethylene oxide caused not only pre- and postimplantation loss but also induced malformations (Generoso et al., '88; Rutledge & Generoso '89). Indeed, preimplantation exposure to cyclophosphamide itself resulted in decreased cell numbers and adverse postimplantation development (Eibs & Spielmann, '77, Kola et al., '86). Prolonged exposure to tritiated thymidine also reduced preimplantation embryo cell numbers (Snow, '73). The inner cell mass was the more sensitive of the two cell lineages to chemical injury by many agents including chronic tritiated thymidine (Snow, '73) and cyclophosphamide (Spielmann et al., '81).

C. Experimental Design

The chronic low-dose treatment which Sprague-Dawley rats receive in this protocol is comparable to human doses used for maintenance chemotherapy (1-2 mg/kg/day; Livingston & Carter, '70). Treatment of male Sprague-Dawley rats was based on previous studies in which a dose of 6 mg/kg/day did not affect survival of the males but provided significant effects on progeny outcome (Trasler et al., '87). Mating after a 4-5 week treatment period resulted in maximal post-implantation loss with insignificant preimplantation loss.

This treatment period was thus ideal for isolation of the postimplantation effects of cyclophosphamide for further study. The cyclophosphamide model has been well characterized in previous studies from this laboratory (Trasler et al., '85;'86;'87;'88; Hales & Robaire '90; Hales et al., '92), and is an excellent model for male-mediated embryotoxic effects.

D. Formulation of the Project

The goal of this thesis was to clarify the nature of the effects of paternal cyclophosphamide exposure on development which led to postimplantation loss. Previous work demonstrated that the primary effect of paternal cyclophosphamide exposure was a dramatic increase in the loss of embryos early after implantation, before day 8 of gestation (unpublished observations). Because of the apparent role of the paternal genes in the development of the extraembryonic tissues, the initial study was undertaken to determine whether or not the effects of paternal cyclophosphamide treatment were tissue-specific in the implantation site on day 7, just prior to embryo resorption. Male Sprague-Dawley rats were given cyclophosphamide (6 mg/kg/day) or saline by gavage, and bred to untreated female rats after 4 weeks of treatment. Pregnant female rats were killed on day 7 of gestation and implantation sites were dissected from the uterus, fixed, embedded in Epon for semi-

thin sectioning, and stained for subsequent light microscopy. Serial sections through entire implantation sites were examined for embryonic and extraembryonic cell morphology.

To approach the triggering event caused by exposure of male germ cells to cyclophosphamide which led to such a dramatic alteration in embryo development, it was important to determine whether embryo development was affected by paternal cyclophosphamide treatment prior to day 7. Thus the next approach was to examine embryos at progressively earlier gestational ages, measuring cell number, a parameter of the developmental stage of the embryo. Male rats were treated as described above and mated to virgin females. Embryos were isolated from the reproductive tracts on day 2, day 2.5, day 3, and day 4 of gestation. Cell numbers were determined by counting fluorescently-stained cell nuclei.

Once the first appearance of a difference in cell number was determined, the third study related cellular DNA synthesis to decreased cell number. Tritiated thymidine uptake was measured in day 3 and day 4 embryos sired by treated and control males, using liquid scintillation spectroscopy to determine cellular DNA synthesis.

Since the sensitivities of the two preimplantation cell lineages may differ, and in order to explain the morphological appearance of the day 7 implantation site, the next experiment was designed to determine if the

preimplantation effects of paternal cyclophosphamide treatment were selective for one cell population. Again males were treated as described and mated to virgin females. Embryos were isolated from females on day 4 of gestation. A differential staining technique with fluorescent chromatin dyes was used to analyze cell numbers in the inner cell mass and trophectoderm lineages of the day 4 blastocyst.

The retarded development of embryos sired by treated males was related to ultrastructure in the final study using transmission electron microscopy. Males were treated as described and mated to virgin females. Embryos were isolated on day 2.5, 3, and 4 of gestation, and processed for electron microscopy. The uniformity of effects on cell morphology and relative stage of development was examined with consideration of possible modes of cell degeneration.

References

Abraham SK, Fränz J. Induction of sister-chromatid exchanges by chemotherapeutic drugs in spermatogonia of mice: effects of procarbazine, adriamycin, cyclophosphamide and mitomycin C. Mutat Res 1983; 108:373-381.

Adams PM, Fabricant JD, Legator MS. Cyclophosphamide-induced spermatogenic effects detected in the $F_1$ generation by behavioral testing. Science 1981; 211:80-82.

Adams PM, Fabricant JD, Legator MS. Active avoidance behaviour in the $F_1$ progeny of male rats exposed to cyclophosphamide prior to fertilization. Neurobehav Toxicol Teratol 1982; 4:531-534.

Auroux MR, Dulioust EM, Nawar NY, Yacoub SG. Antimitotic drugs (cyclophosphamide and vinblastine) in the male rat: deaths and behavioral abnormalities in the offspring. J Androl 1986; 7:378-386.

Auroux MR, Dulioust EJB, Nawar NNY, Yacoub SG, Mayaux MJ, Schwartz D, David G. Antimitotic drugs in the male rat: behavioral abnormalities in the second generation. J Androl 1988; 9:153-159.

Backer LC, Gibson JB, Moses MJ, Aleen JW. Synaptonemal complex damage in relation to meiotic chromosome aberrations after exposure of male mice to

cyclophosphamide. Mutat Res 1988; 203:317-30.

Barlow DP, Stöger R, Herrmann BG, Saito K, Schweifer N. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the *Tme* locus. Nature 1991; 349:84-87.

Barton SC, Surani MAH, Norris ML. Role of paternal and maternal genomes in mouse development. Nature 1984; 311:374-376.

Barton SC, Adams CA, Norris ML, Surani MAH. Development of gynogenetic and parthenogenetic inner cell mass and trophectoderm tissues in reconstituted blastocysts in the mouse. J Embyrol Exp Morphol 1985; 90:267-285.

Baverstock KF. DNA instability, paternal irradiation and leukaemia in children around Sellafield. Int J Radiat Biol 1991; 60:581-95.

Benson AJ, Martin CN, Garner RC. *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl]amine, the putative major DNA adduct of cyclophosphamide *in vitro* and *in vivo* in the rat. Biochem Pharmacol 1988; 37:2979-85.

Brender JD, Suarez L. Paternal occupation and anencephaly. Am J Epidemiol 1990; 131:517-21.

Brewen JG, Payne HS, Jones KP, Preston RJ. Studies on chemically induced dominant lethality. I. The cytogenetic basis of MMS-induced dominant lethality in post-meiotic male germ cells. Mutat Res 1975; 33:239-50.

Byrne J, Mulvihill JJ, Myers MH, Connelly RR, Naughton MD, Krauss MR, Steinhorn SC, Hassinger DD, Austin DF, Bragg K, Holmes GF, Holmes FF, Latourette HB, Weyer PJ, Meigs JW, Teta MJ, Cook JW, Strong LC. Effects of treatment on fertility in long-term survivors of childhood or adolescent cancer. N Engl J Med 1987; 317:1315-21.

Charak BS, Gupta R, Mandrekar P, Sheth NA, Banavali SD, Saikia TK, Gopal R, Dinshaw KA, Advani SH. Testicular dysfunction after cyclophosphamide-vincristine-procarbazine-prednisolone chemotherapy for advanced Hodgkin's disease. Cancer 1990; 65:1903-06.

Chellman GJ, Bus JS, Working PK. Role of epididymal inflammation in the induction of dominant lethal mutations in Fischer 344 rat sperm by methyl chloride. Proc Natl Acad Sci USA 1986; 83:8087-91.

Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 1972; 52:198-236.

DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell 1991; 64:849-59.

Dulioust EM, Nawar NY, Yacoub SG, Ebel AB, Kempf EH, Auroux MR. Cyclophosphamide in the male rat: new pattern of anomalies in the third generation. J Androl 1989; 10:296-303.

Dvorak M. The differentiation of rat ova during cleavage.

Adv Anat Embryol Cell Biol 1978; 55:1-131.

Eibs H-G, Spielmann H. Inhibition of post-implantation development of mouse blastocysts *in vitro* after cyclophosphamide treatment *in vivo*. Nature 1977; 270:54-56.

Ehling UH, Cumming RB, Malling HW. Induction of dominant lethal mutations by alkylating agents in male mice. Mutat Res 1968; 5:417-28.

Fabricant JD, Legator MS, Adams PM. Post-meiotic cell mediation of behavior in progeny of male rats treated with cyclophosphamide. Mutat Res 1983; 119:185-190.

Fairley KF, Barrie JU, Johnson W. Sterility and testicular atrophy related to cyclophosphamide therapy. Lancet 1972; 1:568-569.

Fried P, Steinfeld R, Casileth B, Steinfeld A. Incidence of developmental handicaps among the offspring of men treated for testicular seminoma. Int J Androl 1987; 10:385-7.

Fritz H, Müller D, Hess R. Embryolethality in the mouse following treatment of males with cyclophosphamide at specific germ cell stages. Agents Actions 1973; 3:35-37.

Fukutani K, Ishida H, Shinohara M, Minowada S, Niijima T, Hijikata K, Izawa Y. Suppression of spermatogenesis in patients with Behçet's disease treated with cyclophosphamide and colchicine. Fertil Steril 1981;

36:76-80.

Gardner MH, Snee MP, Hall AJ, Powell CA, Downes S, Terrell JD. Results of a case-control study of leukemia and lymphoma among young people near Sellafield nuclear plant in West Cumbria. Br Med J 1990; 300:423-29.

Gardner RL, Rossant J. Determination during embryogenesis, In: Embryogenesis in Mammals, Ciba Foundation Symposium. Amsterdam: Associated Scientific Publishers; 1976: 5-25.

Generoso WM, Rutledge JC, Cain KT, Hughes LA, Downing DJ. Mutagen-induced fetal anomalies and death following treatment of females within hours after mating. Mutat Res 1988; 395:117-131.

Goldstein LS, Spindle AI. Detection of X-ray induced dominant lethal mutations in mice: an in vitro approach. Mutat Res 1976; 41:289-96.

Hales BF. Comparison of the mutagenicity and teratogenicity of cyclophosphamide and its active metabolites, 4-hydroxycyclophosphamide, phosphoramide mustard, and acrolein. Cancer Res 1982; 42:3016-21.

Hales BF, Crosman K, Robaire B. Increased postimplantation loss and malformations among the $F_2$ progeny of male rats chronically treated with cyclophosphamide. Teratology 1992; 45:671-678.

Hales BF, Robaire B. Reversibility of the effects of chronic paternal exposure to cyclophosphamide on

pregnancy outcome in rats. Mutat Res 1990; 229:129-134.

Hales BF, Robaire B. Paternally mediated effects on progeny outcome. In: Whitcomb RW, Zirkin BR (eds.), Understanding Male Infertility: Basic and clinical approaches. Serono Symposia Publications, vol 98. New York: Raven Press; 1993: 307-320.

Hales BF, Smith S, Robaire B. Cyclophosphamide in the seminal fluid of treated males: transmission to females by mating and effect on pregnancy outcome. Toxicol Appl Pharmacol 1986; 84:423-430.

Jackson H. The effects of alkylating agents on fertility. Brit Med Bull 1964; 20:107-14.

Jenkinson PC, Anderson D. Malformed foetuses and karyotype abnormalities in the offspring of cyclophosphamide and allyl alcohol-treated male rats. Mutat Res 1990; 229:173-184.

Kinlen LJ. Can paternal preconceptional radiation account for the increase of leukaemia and non-Hodgkin's lymphoma in Seascale? Brit Med J 1993; 306:1718-1721.

Kirk KM, Lyon MF. Induction of congenital malformations in the offsping of male mice treated with X-rays at pre-meiotic and post-meiotic stages. Mutat Res 1984; 125:75-85.

Kochhar DM. Limb development in mouse embryos. 1. Analysis of teratogenic effects of retinoic acid. Teratology

1973; 7: 289-298.

Kola I, Folb PI, Parker MI. Maternal administration of cyclophosphamide induces chromosomal aberrations and inhibits cell number, histone synthesis, and DNA synthesis in preimplantation mouse embryos. Teratogenesis Carcinog Mutagen 1986; 6:115-127.

Lähdetie J. Induction and survival of micronuclei in rat spermatids. Comparison of two meiotic micronucleus techniques using cyclophosphamide. Mutat Res 1988; 203:47-53.

Lewis DE. Developmental analysis of lethal effects of homozygosity for the $c^{25H}$ deletion in the mouse. Dev Biol 1978; 65:553-557.

Livingston RB, Carter SK. In: Single Agents in Cancer Chemotherapy. New York: IFI/Plenum; 1970: 25-80.

Lyon MF. Sensitivity of various germ-cell stages to environmental mutagens. Mutat Res 1981; 87:323-45.

Magnuson T. Mutations and chromosomal abnormalities: how are they useful for studying genetic control of early mammalian development? In: Rossant J, Pedersen RA, eds. Experimental Approaches to Early Embryonic Development. New York: Cambridge University Press; 1986: 437-474.

Magnuson T, Epstein CJ. Oligosyndactyly: a lethal mutation in the mouse that results in mitotic arrest very early in development. Cell 1984; 38:823-833.

Magnuson T, Epstein CJ. Genetic expression during early mouse development. In: Bavister BD (ed.), The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation *in Vitro*. New York: Plenum Press; 1987: 133-150.

Matsuda Y, Tobari I, Maemori M, Seki N. Mechanism of chromosome aberration induction in the mouse egg fertilized with sperm recovered from postmeiotic germ cells treated with methyl methanesulfonate. Mutat Res 1989; 214:165-80.

McDonald AD, McDonald JC, Armstrong B, Cherry NM, Nolin AD, Robert D. Fathers' occupation and pregnancy outcome. Br J Ind Med 1989; 46:329-33.

McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 1984; 37:170-183.

Meistrich ML, Finch M, Da Cunha MF, Hacker U, Au WW. Damaging effects of fourteen chemotherapeutic drugs on mouse testis cells. Cancer Res 1982; 42:122-31.

Moreland FM, Sheu CW, Springer JA, Green S. Effects of prolonged chemical treatment with cyclophosphamide and 6-mercaptopurine in the dominant lethal test system. Mutat Res 1981; 90:193-199.

Nadijcka MD, Hillman N, Gluecksohn-Waelsch S. Ultrastructural studies of lethal $c^{25H}/c^{25H}$ mouse embryos. J Embryol Exp Morphol 1979; 52:1-11.

O WS, Chen HQ, Chow PH. Effects of male accessory sex gland secretions on early embryonic development in the golden hamster. J Reprod Fert 1988; 84:341-44.

Olshan AF, Teschke K, Baird PA. Birth defects among offspring of firemen. Am J Epidemiol 1990; 131:312-21.

Olshan AF, Teschke K, Baird PA. Paternal occupation and congential anomalies. Am J Ind Med 1991; 20:447-75.

Orgebin-Crist M-C. Studies on the function of the epididymis. Biol Reprod 1969; 1:155-175.

Pacchierotti F, Bellincampi D, Civitareale D. Cytogenetic observations, in mouse secondary spermatocytes, on numerical and structural chromosome aberrations induced by cyclophosphamide in various stages of spermatogenesis. Mutat Res 1983; 119:177-83.

Pampfer S, Streffer C. Prenatal death and malformations after irradiation of mouse zygotes with neutrons or X-rays. Teratology 1988; 37:599-607.

Pampfer S, Vanderheyden I, Michiels B, De Hertogh R. Cell allocation to the inner cell mass and the trophectoderm in rat embryos during in vivo preimplantation development. Roux's Arch Dev Biol 1990; 198:257-263.

Pedersen RA. Development of lethal yellow ($A^y/A^y$) mouse embryos in vitro. J exp Zool 1974; 188:307-320.

Pedersen RA. Potency, lineage, and allocation in preimplantation mouse embryos. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian

Embryonic Development. New York: Cambridge University Press; 1986: 3-33.

Qiu J, Hales BF, Robaire B. Effects of cyclophosphamide on sperm nuclear proteins. Manuscript in preparation.

Qureshi MSA, Pennington JH, Goldsmith HJ, Cox PE. Cyclophosphamide therapy and sterility. Lancet 1972; 2:1290-1291.

Rathenberg R, Müller D. Comparative cytogenetic studies of the influence of phenylbutazone and cyclophosphamide on spermatogenesis in the mouse. Agents Actions 1972; 2:180-185.

Robarie B, Hales BF. Paternal exposure to chemicals before conception: some children may be at risk. Brit Med J 1993; 307:341-342.

Robaire B, Hermo L. Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neill J, et al. (eds.), The Physiology of Reproduction. New York:Raven Press Ltd.; 1988: 999-1080.

Robaire B, Trasler JM, Hales BF. Consequences to the progeny of paternal drug exposure. In: Lobl TJ, Hafez ESE (eds.), Male Fertility and its Regulation. Boston: MTP Press Ltd; 1985:225-43.

Rossant J, Vijh M, Siracusa LD, Chapman VM. Identification of embryonic cell lineages in histological sections of *M. musculus* <--> *M. caroli* chimaeras. J Embryol Exp

Morphol 1983; 73:179-191.

Rowley MJ, Leach DR, Warner GA, Heller CG. Effect of graded doses of ionizing radiation on the human testis. Radiat Res 1974; 59:665-78.

Rutledge JC, Cain KT, Kyle J, Coornett CV, Cacheiro NLA, Witt K, Shelby MD, Generoso WM. Increased incidence of developmental anomalies among descendants of carriers of methylenebisacrylamide-induced balanced reciprocal translocations. Mutat Res 1990; 229:161-72.

Rutledge JC, Generoso WM. Fetal pathology produced by ethylene oxide treatment of the murine zygote. Teratology 1989; 39:563-572.

Savitz DA, Whelan EA, Kleckner RC. Effect of parents' occupational exposures on risk of stillbirth, preterm delivery, and small-for-gestational-age infants. Am J Epidemiol 1989; 129:1201-18.

Schmahl D, Habs M. Carcinogenic action of low-dose cyclophosphamide given orally to Sprague-Dawley rats in a lifetime experiment. Int J Cancer 1979; 23:706-12.

Schultz GA, Heyner, S. Gene expression in pre-implantation mammalian embryos. Mutat Res 1992; 296:17-31.

Sega GA. Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulfonate. Proc Natl Acad Sci USA 1974; 71:4955-4959.

Senturia YD, Peckham CS, Peckham MJ. Children fathered by

men treated for testicular cancer. Lancet 1985; ii:766-9.

Snow MHL. Abnormal development of pre-implantation mouse embryos grown in vitro with [$^3$H]-thymidine. J Embryol exp Morph 1973; 29:601-615.

Sotomayor RE, Cumming RB. Induction of translocations by cyclophosphamide in different germ cell stages of male mice: cytological characterization and transmission. Mutat Res 1975; 27:375-388.

Spielmann H, Jacob-Muller U, Eibs H-G, Beckford W. Investigations on cyclophosphamide treatment during the preimplantation period I. Differential sensitivity of preimplantation mouse embryos to maternal cyclophosphamide treatment. Teratology 1981; 23:1-5.

Sprinks MT, Sellens MH, Dealtry GB, Fernandez N. Preimplantation mouse embryos express Mhc class I genes before the first cleavage division. Immunogenetics 1993; 38:35-40.

Surani MAH. Evidence and consequences of differences between maternal and paternal genomes during embryogenesis in the mouse. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian Embryonic Development. New York: Cambridge University Press; 1986: 401-435.

Surani MAH, Barton SC, Norris ML. Development of reconstituted mouse eggs suggests imprinting of the

genome during gametogenesis. Nature 1984; 316:144-146.

Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 1990; 26:90-100.

Trasler JM, Hales BF, Robaire B. Paternal cyclophosphamide treatment of rats causes fetal loss and malformations without affecting male fertility. Nature 1985; 316:144-146.

Trasler JM, Hales BF, Robaire B. Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. Biol Reprod 1986; 34:275-283.

Trasler JM, Hales BF, Robaire B. A time-course study of chronic paternal cyclophosphamide treatment in rats: effects on pregnancy outcome and the male reproductive and hematologic systems. Biol Reprod 1987; 37:317-326.

Trasler JM, Hermo L, Robaire B. Morphological changes in the testis and epididymis of rats treated with cyclophosphamide: a quantitative approach. Biol Reprod 1988; 38:463-79.

Trasler JM, Robaire B. Effects of cyclophosphamide on selected cytosolic and mitochondrial enzymes in the epididymis of the rat. J Androl 1988; 9:142-52.

Van Buul PPW. Enhanced radiosensitivity for the induction of translocations in mouse stem cell spermatogonia

following treatment with cyclophosphamide or adriamycin. Mutat Res 1984; 128:207-211.

Velez de la Calle JF, de Queiroz F, Garnier DH, Kercret H, Folliot R, Jégou B. Reproductive effects of the anticancer drug cyclophosphamide in male rats at different ages. Arch Androl 1989; 22:251-263.

Wiley LM. What is the radiosensitive target of mammalian gametes and embryos at low doses of radiation? In: Allen JW, Bridges BA, Lyon MF, Moses MJ, Russell LB, (eds.), Banbury Report 34: Biology of Mammalian Germ Cell Mutagenesis. New York: Cold Spring Harbor Laboratory Press; 1990: 299-310.

Wilson, JG. Current status of teratology - general principles and mechanisms derived from animal studies. In: Wilson JG, Fraser FC, (eds.), Handbook of Teratology. 1. General Principles and Etiology. New York: Plenum Press; 1977:47-74.

Wudl LR, Sherman MI, Hillman N. Nature of lethality of t mutations in embryos. Nature 1977; 270:137-140.

Chapter II

# Paternal cyclophosphamide treatment causes postimplantation loss via inner cell mass-specific cell death.

Sara M. Kelly, Bernard Robaire, and Barbara F. Hales

ABSTRACT

Treatment of the father with the anticancer alkylating agent cyclophosphamide has negative effects on embryonic development in the rat. Four-week treatment of male rats with a low dose of cyclophosphamide causes a dramatic, dose-dependent increase in postimplantation death of the progeny. Several recent studies have indicated that the paternal genome is required for the development of the extra-embryonic tissues. Thus, the purpose of this study was to determine which tissues of the implanting embryo were affected by paternal exposure to cyclophosphamide. Male Sprague-Dawley rats were given cyclophosphamide (6 mg/kg/day) or saline by gavage, and bred to untreated female rats after 4 weeks of treatment. Pregnant female rats were killed on day 7 of gestation and implantation sites were dissected from the uterus, fixed, embedded in Epon for semi-thin serial sectioning, and stained for subsequent light microscopy. Strikingly, many of the implantation sites of affected embryos sired by treated males displayed an apparently normal trophectoderm enclosing a region of dying cells, containing darkly stained pycnotic nuclei. Very few or no inner cell mass-derived embryonic cells were present in these implantation sites. Therefore, there is a selective death of inner cell mass-derived cells in day 7 implantation sites obtained from the progeny of

cyclophosphamide-treated males. The results of this study suggest that treatment of the male with cyclophosphamide can affect paternal genes specifically required for development of the inner cell mass cells of the embryo, without an apparent effect on those genes required for normal trophectoderm.

# INTRODUCTION

The principal target of many of the chemotherapeutic drugs which act as teratogens is thought to be DNA; alterations in DNA are hypothesized to lead to changes in the developmental program of the embryo. Since one half of the genetic material in an embryo is of paternal origin, the genetic material derived from the father should be as significant, and potentially as susceptible to insult, as that from the mother. This conclusion has been supported by the work of McGrath and Solter ('84) and Surani (Barton et al.,'84; '85; Surani et al., '84), which demonstrated an absolute requirement for the pronuclei from both parents for normal development of mouse embryos.

Cyclophosphamide is a commonly used anticancer and immunosuppressive agent which is administered in acute doses for cancer chemotherapy and chronic low doses for maintenance chemotherapy and immunosuppression. Cyclophosphamide is carcinogenic (Schmal and Habs, '79), mutagenic (Hales, '82), and teratogenic (Gibson and Becker, '68; Hales, '82). The teratogenic effects of maternal cyclophosphamide treatment on the embryo during gestation are well documented (Chaube et al., '67; Porter and Singh, '88). In men undergoing chemotherapy, it has been known for some time that cyclophosphamide causes azoospermia and oligospermia (Fairley et al., '72; Qureshi et al., '72;

Fukutani, '81).

There have been a number of studies showing an effect of cyclophosphamide treatment on spermatogenesis and progeny outcome (Rathenberg and Muller, '72; Sotomayor and Cumming, '75; Adams et al., '81; Velez de la Calle et al., '89; Jenkinson and Anderson, '90). Previous work in this laboratory characterized the effect of cyclophosphamide on progeny outcome when given in chronic low doses. There was a dramatic time- and dose-dependent increase in early postimplantation loss without a major effect on the male reproductive system (Trasler et al., '85, '86, '87). A four-week exposure of the male germ cells to low doses of cyclophosphamide resulted in the death of more than 80% of the progeny around the time of implantation.

The mechanism by which exposure of male germ cells to cyclophosphamide causes the death of the embryo at a specific period in gestation is presently unclear. In studies of parental contributions to embryonic development (McGrath and Solter, '84; Barton et al., '84; '85; Surani et al., '84), embryos lacking a paternal genome were particularly deficient in extra-embryonic tissues. The present study was designed to assess whether exposure of the paternal genome to cyclophosphamide might have a similar negative effect on extra-embryonic tissues in the implanting embryo. The results show that chronic paternal treatment with cyclophosphamide has a highly specific lethal effect

not on the extra-embryonic tissues but on the inner cell mass lineage of embryonic cells. This is, we believe, the first demonstration of lineage-specific cell death in the peri-implantation embryo after paternal drug exposure.

# MATERIALS AND METHODS

## Treatment of males and mating schedule.

Male (300-350 g) and virgin female (225-250 g) Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, Québec). Animals were maintained on a 14 hr L:10 hr D cycle and provided with food and water ad libitum. Male rats were randomly divided into two groups of six rats each (control and cyclophosphamide-treated). Males were gavaged as previously described (Trasler et al., '86). Treated males received 6.0 mg/kg/day cyclophosphamide and control males received an equal volume of saline. On the seventh day of week 4 of the treatment schedule, males were not gavaged, but were mated overnight with two female rats in proestrus. Females were examined the morning after mating for the presence of spermatozoa in the vagina (day 0 = morning after mating when spermatozoa are present in the vaginal smear).

### *Implantation sites*

Pregnant females were killed on day 7 of gestation and decidual swellings were isolated from explanted uterine tissue. Implantation sites were fixed for 2 hr in 2.5% glutaraldehyde, washed, treated with osmium, and serially dehydrated for embedding in Epon. Approximately five implantation sites sired by four of the males from each group were randomly chosen for sectioning. Each flat-

embedded implantation site was serially sectioned (7 μm thick sections) longitudinally and stained with toluidine blue. Sections were examined by light microscopy. For analysis of differentiated cell types, the widest section through the implantation cylinder was selected from each implantation site.

*Statistical Analysis*

Data were analyzed by Fisher's exact test (Zar, '74), with an IBM PC computer using the CSS (Complete Statistical System) software program (Statsoft Inc., Tulsa, OK). The level of significance was $P \leq 0.05$.

## RESULTS

Randomly-selected implantation sites were examined in a blinded analysis for the presence or absence of trophectoderm and inner cell mass-derived cells. Three inner cell mass assessment categories, inner cell mass-positive, inner cell mass-retarded, and inner cell mass-negative, were established.

The inner cell mass-positive implantation sites displayed normal trophectoderm cells interfacing with the maternal decidual cells (Fig. 1a). The ectoplacental cone connected with the parallel layers of extra-embryonic ectoderm and endoderm. These rows of cells abutted the embryonic portion of the implanting embryo, the two

semicircular layers of embryonic cells. The more darkly-staining embryonic ectoderm was surrounded by the embryonic endoderm layer (Fig. 1a,b). The incidence of cell death among the embryonic cells was very low (less than 10%).

The inner cell mass-retarded implantation sites displayed an apparently normal trophectoderm, enclosing a region of small apparently undifferentiated cells and cell fragments (Fig. 1c,d). The diameter of the implantation cylinder was dramatically reduced and no recognizable embryonic structures were visible.

Implantation sites which were inner cell mass-negative contained apparently normal trophectoderm cells in contact with normal maternal decidual cells (Fig. 1e,f). Although the implantation cylinder extended the full length of the implantation site, only small fragments of debris were visible in the narrow space between the walls. There were no recognizable structures of any kind in these implantation sites except for the trophectoderm.

There was no apparent effect of paternal drug exposure on the trophectoderm cells (Fig. 2). In contrast to this lack of an effect on the trophectoderm, there was a dramatic shift from inner cell mass-positive to both severely depleted inner cell mass-retarded ($P = 0.002$) and inner cell mass-negative ($P = 0.01$) embryos in the majority of implantation sites after paternal treatment with cyclophosphamide (Fig. 2). This increased incidence of

implantation sites with an affected inner cell mass (84%) is similar to the increased postimplantation loss in previous studies after four weeks of treatment at a similar dose (Trasler et al., '86, '87). Thus, cyclophosphamide can selectively alter the male germ cell so that in the embryos arising from these germ cells, the inner cell mass-derived cells die around the time of implantation.

# DISCUSSION

Treatment of male rats with cyclophosphamide for four weeks exposes the male germ cells to the drug in the testis from the early spermatid stage of spermatogenesis to the mature spermatozoal stage in the epididymis (Clermont, '72). The present study shows that this treatment produces a dramatic increase in inner cell mass-negative and retarded implantation sites.

The early spermatid is the product of the second meiotic division in spermatogenesis (Clermont, '72). Although it has been proposed that the spermatogonia are most susceptible to cell killing by chemotherapeutic drugs (Meistrich, '86), spermatozoa and spermatids are the germ cells most susceptible to cyclophosphamide-induced dominant lethal mutations (Ehling and Neuhäuser-Klaus, '88). In fact, like cyclophosphamide, other alkylating agents such as ethyl or methyl methanesulfonate, chlormethine or treatment with X-rays affect spermatogenesis and/or progeny outcome with their greatest effect on post-meiotic germ cells (Ehling et al., '68; Jackson, '64; Kirk and Lyon, '84; Ehling and Neuhäuser-Klaus, '89a; 89b; '90). Early to mid-spermatids do show unscheduled DNA synthesis, but late spermatids and spermatozoa do not undergo unscheduled DNA synthesis or DNA repair until they enter the fertilized egg (Sega, '74). This lack of the ability to repair damage may

contribute to increase the susceptibility of the more mature germ cell stages to alkylating agents, and occurs in germ cell stages where protamine has replaced the chromosomal histones (Sega, '74).

There are two potential molecular targets, DNA and proteins, in the spermatid which can be alkylated by active metabolites of cyclophosphamide (Foley et al., '61). Indeed, the active metabolites of cyclophosphamide, phosphoramide mustard and acrolein, have been shown previously to alkylate both DNA (Benson et al., '88) and protein (Mirkes and Little, '90), and to cause chromosome breaks and translocations in male germ cells (Sotomayor and Cumming, '75).

Although spermatid proteins may be alkylated by metabolites of cyclophosphamide, it is unlikely that these alkylated proteins would persist until day 7 of gestation in the fertilized embryo. Nevertheless, it is possible that alkylated proteins could convey their damage to the genetic material of the spermatid. As the spermatids mature, the nuclei condense in a specific pattern, forming increasingly electron-dense and tightly-packed bundles of chromatin (Lalli and Clermont, '81; Ward and Coffey, '91). Although most alkylating agents are non-specific, the DNA of the spermatid may be undergoing folding or condensing processes which make certain regions of the genome selectively vulnerable to alkylation. Whether the alterations in the

male germ cell after exposure to cyclophosphamide are genetic or epigenetic in nature remains to be determined. What is clear, however, is that the change in the genetic material of the spermatozoa after cyclophosphamide treatment has dramatic consequences for the embryo.

To have such a marked effect on the implantation site, the contribution made by the cyclophosphamide-treated paternal genome to the embryo must differ in some way from the contribution of an unexposed paternal genome. The imprinting studies described by Surani ('86) indicated that the paternal contribution appeared to be essential for proliferation of the extra-embryonic tissues. We thus expected to find the extra-embryonic tissues more affected when the paternal contribution to the embryonic genome was damaged by cyclophosphamide treatment. The normal appearance of the trophectoderm indicates that the expression of those paternal genes required for development of extra-embryonic tissue apparently was not affected by cyclophosphamide treatment. The selective effect on inner cell mass-derived tissues indicates that there are paternal genes expressed in the inner cell mass which can be lethally affected by cyclophosphamide treatment. It is also possible that there may be differences in the abilities of inner cell mass and trophectoderm-derived cells to repair DNA damage and chromosomal breaks.

There is clear evidence that DNA rearrangements can

lead to early postimplantation embryo lethality. The integration of plasmid DNA into the genome of microinjected zygotes has been reported to lead to DNA rearrangements in transgenic mice which result in postimplantation developmental arrest (Covarrubias et al., '86; Mahon et al., '88). Interestingly, the embryo lethality in these transgenic mouse lines occurred in the egg cylinder stage and was characterized by empty decidua. A singular genetic defect also can cause an inner cell mass-specific defect in the implanting embryo. The radiation-induced mutation oligosyndactyly (*Os*) in the mouse causes skeletal and muscular defects as well as nephrogenic diabetes insipidus in heterozygous mice (Magnuson, '86). In the homozygous offspring, however, the inner cell mass cells degenerate while the trophectoderm-derived cells remain apparently viable (Magnuson and Epstein, '84). While this defect is only seen when both parental chromosomes carry the mutation, it establishes a precedent for the ability of a singular mutation to cause death in inner cell mass-derived cells at a very specific time in gestation.

The early postimplantation loss seen in the present study may have serious implications for human male infertility after chemotherapy. The sperm of males who are diagnosed as infertile after chemotherapy may indeed be capable of fertilizing embryos, but may produce embryos which die immediately after implantation.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the helpful suggestions and expertise of Dr. E. Daniels, Dr. A. da Silva, and Ms. M. Ballak. This work was supported by the Medical Research Council of Canada.

## References


Adams, P.M., J.D. Fabricant, and M.S. Legator (1981) Cyclophosphamide-induced spermatogenic effects detected in the $F_1$ generation by behavioral testing. Science, *211*:80-82.

Barton, S.C., M.A.H. Surani, and M.L. Norris (1984) Role of paternal and maternal genomes in mouse development. Nature, *311*:374-376.

Barton, S.C., C.A. Adams, M.L. Norris, and M.A.H. Surani (1985) Development of gynogenetic and parthenogenetic inner cell mass and trophectodern tissues in reconstituted blastocysts in the mouse. J. Embryol. exp. Morph., *90*:267-285.

Benson, A.J., C.N. Martin, and R.C. Garner (1988) *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl]amine, the putative major DNA adduct of cyclophosphamide *in vitro* and *in vivo* in the rat. Biochem. Pharmacol., *37*:2979-2985.

Chaube, S., G. Kury, and M.L. Murphy (1967) Teratogenic effects of cyclophosphamide (NSC-26271) in the rat. Cancer Chemother. Rep., *51*:363-379.

Clermont, Y. (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol. Rev., *52*:198-236.

Covarrubias, L., Y. Nishida, and B. Mintz (1986) Early

postimplantation embryo lethality due to DNA rearrangements in a transgenic mouse strain. Proc. Nat. Acad. Sci. USA, *83*:6020-6024.

Ehling, U.H., R.B. Cumming, and H.V. Malling (1968) Induction of dominant lethal mutations by alkylating agents in male mice. Mutation Res., *5*:417-428.

Ehling, U.H., and A. Neuhäuser-Klaus (1990) Induction of specific-locus and dominant lethal mutations in male mice in the low dose range by methyl methanesulfonate (MMS). Mutation Res., *230*:61-70.

Ehling, U.H., and A. Neuhäuser-Klaus (1989a) Induction of specific-locus mutations in male mice by ethyl methanesulfonate (EMS). Mutation Res., *227*:91-95.

Ehling, U.H., and A. Neuhäuser-Klaus (1989b) Induction of specific-locus and dominant lethal mutations in male mice by chlormethine. Mutation Res., *227*:81-89.

Ehling, U.H., and A. Neuhäuser-Klaus (1988) Induction of specific-locus and dominant-lethal mutations by cyclophosphamide and combined cyclophosphamide-radiation treatment in male mice. Mutation Res., *199*:21-30.

Fairley, K.F., J.U. Barrie, and W. Johnson (1972) Sterility and testicular atrophy related to cyclophosphamide therapy. Lancet, *1*:568-569.

Foley, G.E., O.M. Friedman, and B.P. Drolet (1961) Studies on the mechanism of action of cytoxan - evidence of

activation in vivo and in vitro. Cancer Res., *21*:57-63.

Fukutani, K., H. Ishida, M. Shinohara, S. Minowada, T. Niijima, K. Hijikata, and Y. Izawa (1981) Suppression of spermatogenesis in patients with Behcet's disease treated with cyclophosphamide and colchicine. Fertil. Steril., *36*(1):76-80.

Gibson, J.E., and B.A. Becker (1968) Effect of phenobarbital and SKF 525-A on the teratogenicity of cyclophosphamide in mice. Teratology, *1*:393-398.

Hales, B.F. (1982) Comparison of the mutagenicity and teratogenicity of cyclophosphamide and its active metabolites, 4-hydroxycyclophosphamide, phosphoramide mustard, and acrolein. Cancer Res., *42*:3016-3021.

Jackson, H. (1964) The effects of alkylating agents on fertility. Brit. Med. Bull., *20*:107-114.

Jenkinson, P.C., and D. Anderson (1990) Malformed fetuses and karyotype abnormalities in the offspring of cyclophosphamide and allyl alcohol-treated male rats. Mutation Res., *229*:173-184.

Lalli, M., and Y. Clermont (1981) Structural changes of the head components of the rat spermatid during late spermiogenesis. Am. J. Anat., *160*:419-434.

Magnuson, T. (1986) Mutations and chromosomal abnormalities: How are they useful for studying genetic control of early mammalian development? In: Experimental Approaches to Early Embryonic Development. J. Rossant

and R.A. Pederson, eds. Cambridge University Press. pp. 437-474.

Magnuson, T., and C.J. Epstein (1984) Oligosyndactyly: A lethal mutation in the mouse that results in mitotic arrest very early in development. Cell, *38*:823-833.

Mahon, K.A., P.A. Overbeek, and H. Westphal (1988) Prenatal lethality in a transgenic mouse line is the result of a chromosomal translocation. Proc. Nat. Acad. Sci. USA, *85*:1165-1168.

McGrath, J., and D. Solter (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell, *37*:170-183.

Meistrich, M.L. (1986) Critical components of testicular function and sensitivity to disruption. Biol. Reprod., *34*:17-28.

Mirkes, P.E., and S.A. Little (1990) Binding of (chloroethyl $^{3}H$) cyclophosphamide to rat embryo proteins: two-dimensional gel analysis. Teratology, *41*:195-204.

Porter, A.J., and S.M. Singh (1988) Transplacental teratogenesis and mutagenesis in mouse fetuses treated with cyclophosphamide. Teratogenesis Carcinog. Mutagen., *8*:191-203.

Qureshi, M.S.A., J.H. Pennington, H.J. Goldsmith, and P.E. Cox (1972) Cyclophosphamide therapy and sterility. Lancet, *2*:1290-1291.

Rathenberg, R., and D. Muller (1972) Comparative cytogenetic

studies of the influence of phenylbutazone and cyclophosphamide on spermatogenesis in the mouse. Agents Actions, *2*:180-185.

Schmahl, D., and M. Habs (1979) Carcinogenic action of low-dose cyclophosphamide given orally to Sprague-Dawley rats in a lifetime experiment. Int. J. Cancer, *23*:706-712.

Sotomayor, R.E., and R.B. Cumming (1975) Induction of translocations by cyclophosphamide in different germ cell stages of male mice: cytological characterization and transmission. Mutation Res., *27*:375-388.

Sega, G.A. (1974) Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulfonate. Proc. Nat. Acad. Sci. USA, *71*:4955-4959.

Surani, M.A.H. (1986) Evidences and consequences of differences between maternal and paternal genomes during embryogenesis in the mouse. In: Experimental approaches to mammalian embryonic development. J. Rossant and R.A. Pedersen, eds. Cambridge University Press, New York, pp.401-435.

Surani, M.A.H., S.C. Barton, and M.L. Norris (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature, *308*:548-550.

Trasler, J.M., B.F. Hales, and B. Robaire (1985) Paternal

cyclophosphamide treatment causes fetal loss and malformations without affecting male fertility. Nature, *316*:144-146.

Trasler J.M., B.F. Hales, and B. Robaire (1986) Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. Biol. Reprod., *34*:276-283.

Trasler, J.M., B.F. Hales, and B. Robaire (1987) A time course study of chronic paternal cyclophosphamide treatment of rats: effects on pregnancy outcome and the male reproductive and haematologic systems. Biol. Reprod., *37*:317-326.

Velez de la Calle, J.F., F. de Queiroz, D.H. Garnier, H. Kercret, R. Folliot, and B. Jégou (1989) Reproductive effects of the anticancer drug cyclophosphamide in male rats at different ages. Arch. Androl., *22*:251-263.

Ward, W.S. and D.S. Coffey (1991) DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol. Reprod., *44*:569-574.

Zar, J.H. (1974) Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

Fig. 1 Day 7 implantation site histology. Typical inner cell mass-positive (a,b), inner cell mass-retarded (c,d), and inner cell mass-negative (e,f) implantation sites. Implantation sites were obtained from pregnant female rats after mating with control (a,b), or cyclophosphamide treated male rats (c,d,e,f) groups. The maternal decidual cells (d), trophectoderm cells (arrows), inner cell mass-derived cells and/or cellular debris (arrowheads), extra-embryonic ectoderm (EC), extra-embryonic endoderm (EN), embryonic ectoderm (ec), and embryonic endoderm (en) are indicated. Boxes in (a,c,e) indicate areas enlarged in (b,d,f) respectively. Bars indicate 100$\mu$m.

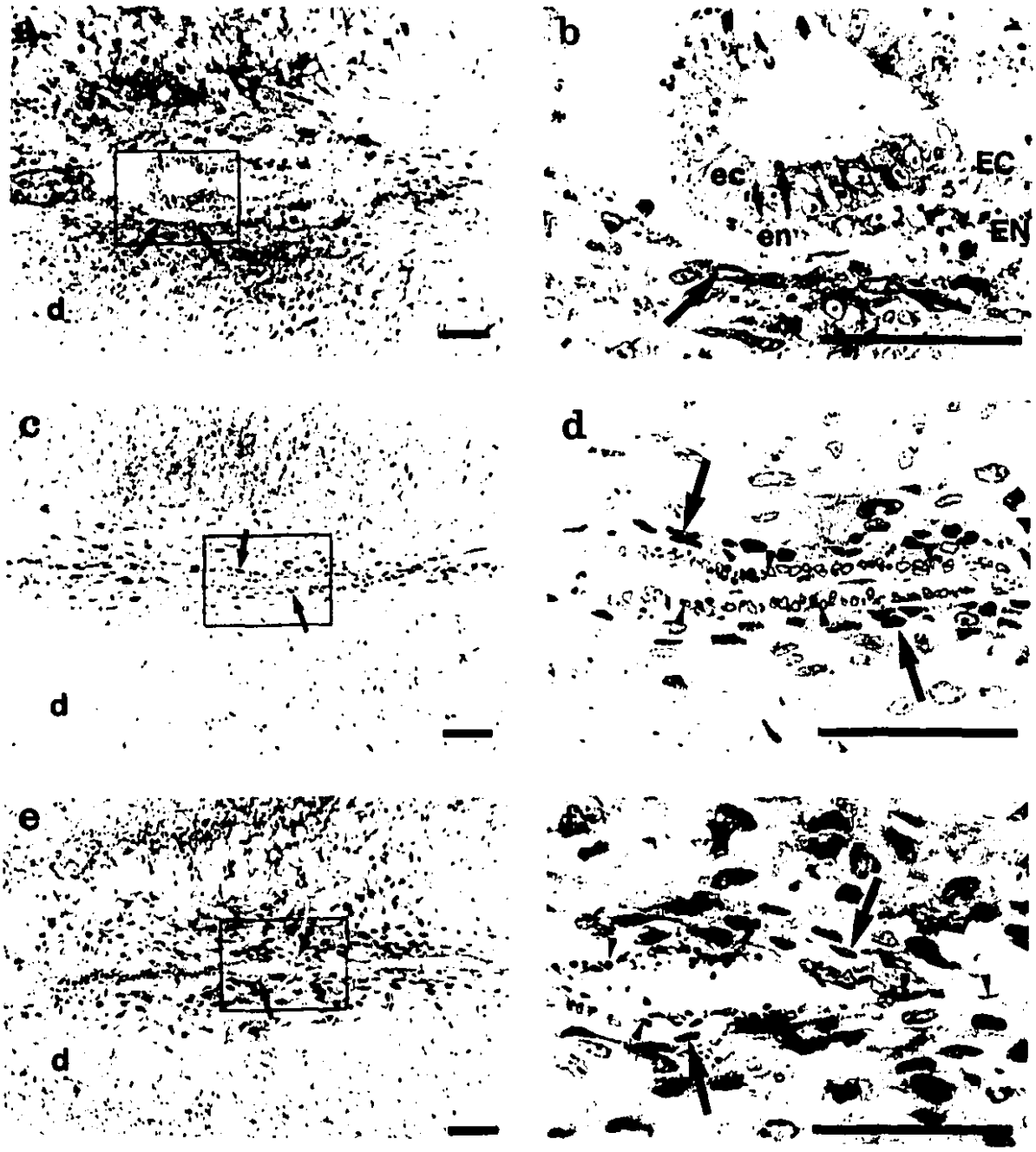
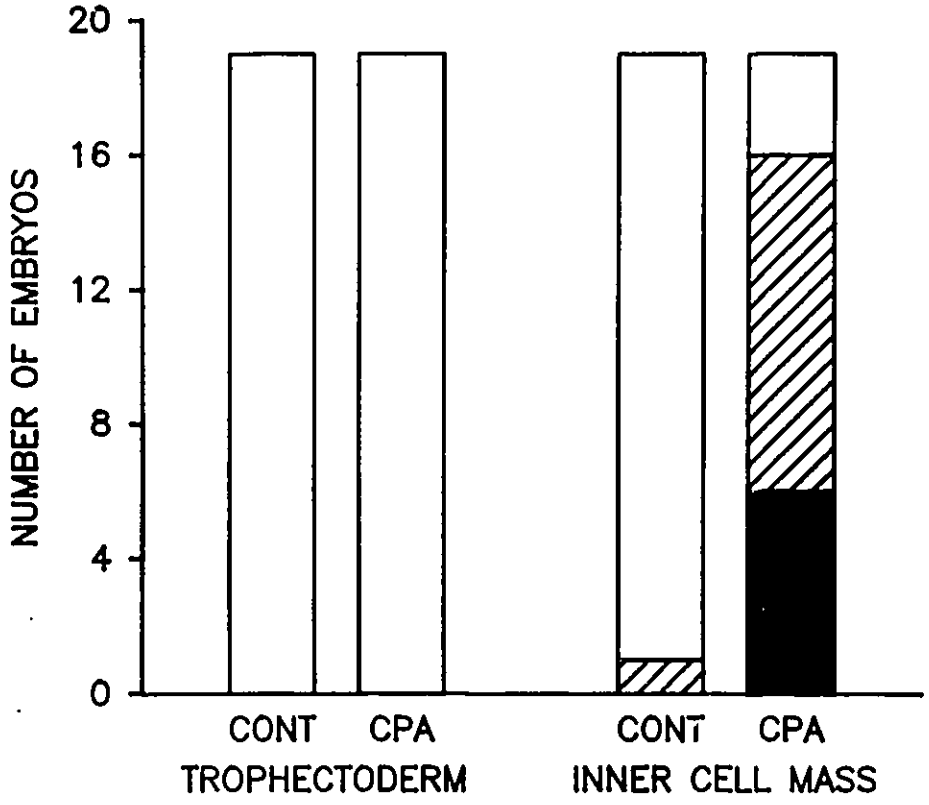

a
d
b
ec
EC
en
EN
c
d
d
e
d

Fig. 2 Effects of four-week paternal cyclophosphamide treatment on the histology of the implantation sites. The number of embryos is expressed as a function of the cell type assessed and the treatment group. Histological appearance was assessed as positive (open bars), retarded (diagonal lines), or negative (solid bars), as illustrated in Figure 1, for the cell lineage in question. Control and treated groups were compared using a Fisher exact test. There was no effect of cyclophosphamide treatment (CPA) compared to controls (CONT) on the trophectoderm cells (open bars). Cyclophosphamide treatment produced a dramatic increase in inner cell mass-retarded ($P = 0.002$) and inner cell mass-negative ($P = 0.01$) implantation sites.


20
16
12
8
4
0
NUMBER OF EMBRYOS
CONT
CPA
CONT
CPA
TROPHECTODERM
INNER CELL MASS

## Connecting Text - Chapter II to Chapter III

The experiments in Chapter II demonstrated that paternal cyclophosphamide exposure resulted in an apparently inner cell mass-specific defect at the peri-implantation stage of development. On day 7 of gestation, the inner cell mass-derived embryonic cells were retarded or absent while trophectoderm-derived extraembryonic cells were apparently normal. The experiments in the next chapter were designed to determine whether this effect in post-implantation embryos occurred as a sudden event after implantation or was a graded effect which began earlier in gestation. In chapter III, preimplantation embryos sired by cyclophosphamide-treated males were examined to determine if development was defective prior to implantation, and if so, whether or not such effects were similarly specific to the inner cell mass cell lineage.

Chapter III

# Paternal cyclophosphamide exposure causes decreased cell proliferation in cleavage-stage embryos

SARA M. KELLY, BERNARD ROBAIRE, and BARBARA F. HALES

## Abstract

Exposure of the male germ cell to cyclophosphamide during spermatogenesis and sperm maturation can interfere with embryo development. When male rats were treated with a chronic low dose of cyclophosphamide for four weeks there was a dramatic increase in early post-implantation loss in their progeny, characterized by implantation sites selectively lacking in embryonic tissues. The present study was designed to determine the earliest appearance of a paternal effect of cyclophosphamide treatment, and to examine whether the embryonic lineage was selectively affected. Male Sprague-Dawley rats were orally dosed for 4-5 weeks with saline or 6 mg/kg/day of cyclophosphamide; their progeny were obtained on days 2, 2.5, 3, 4, and 4.5 of gestation. Paternal cyclophosphamide treatment had no effect on the mean number of embryos per pregnant female. However, there was a significant decrease in cell number among the embryos sired by cyclophosphamide-treated males as early as day 3 of gestation, increasing to a greater than 50% decrease in cell number by day 4. The cell doubling time in embryos sired by treated males (16 h) was longer than that of controls (12 h). This decreased proliferation rate was confirmed by a dramatic decrease in the capacity of both day 3 and day 4 embryos sired by cyclophosphamide-treated males to incorporate [$^{3}$H]-thymidine over a 26-hour

culture period. Cytogenetic analysis in a limited number of blastomeres entering metaphase revealed no evidence of chromosomal abnormalities. Both the trophectoderm and the inner cell mass cells were proportionally decreased in day 4.5 embryos sired by cyclophosphamide-treated males. Thus, paternal cyclophosphamide exposure affected both cell lineages in the conceptus as early as day 3 of gestation.

## Introduction

Functional maternal and paternal genomes are fundamental to the complex genetic signalling events involved in early embryogenesis. Changes in the DNA of either parent as a result of drug exposure may lead to changes in the progression and coordination of development. Cyclophosphamide is an alkylating agent which is commonly used in cancer chemotherapy in high doses for acute treatment and in chronic low doses for maintenance therapy. In men undergoing chemotherapy, cyclophosphamide can cause azoospermia and oligozoospermia (1-3). The deleterious effects of cyclophosphamide on spermatogenesis and progeny outcome have been well documented (4-8). Cyclophosphamide has gross effects on male germ cell chromosomes, causing sister-chromatid exchange (9), translocations (6), and micronucleus formation (10). A number of studies have shown that cyclophosphamide is mutagenic in the rat dominant lethal test system (11) and can cause heritable phenotypic and behavioral defects in offspring; these defects may persist for several generations (12-18). Previous work from our laboratory showed that chronic exposure of males to cyclophosphamide caused increases in pre- and post-implantation loss and in growth retarded and malformed fetuses (19-21). Chronic low doses of cyclophosphamide for four weeks caused greater than 80% early post-implantation loss without major effects on the male reproductive system

(21). In fact, the effect on embryonic post-implantation loss was reversible within four weeks of cessation of treatment (22), implying an effect on the post-meiotic germ cells.

Exposure of the paternal germ cell to cyclophosphamide interferes in a lethal manner with the developmental program of the embryo, however the mechanism(s) has not been identified. Rat embryonic cells begin to differentiate at the 8-cell stage into two cell lineages, the inner cell mass and the trophectoderm, which are clearly discernible at the expanded blastocyst stage. The inner cell mass cells have been traced in cell lineage studies and form predominantly the embryonic tissues of the conceptus, while the trophectoderm cells form predominantly extra-embryonic tissues (23).

Previous studies of parental contributions to embryonic development indicate that the absence of paternal genes results in embryos lacking extra-embryonic tissues, hence paternal genes are important for development of these tissues (24-26). We have shown, however, that dying embryos sired by cyclophosphamide-treated males are selectively deficient in embryonic tissues rather than in extra-embryonic tissues (27). Therefore paternal cyclophosphamide exposure appeared to affect paternal genes required for the development of the inner cell mass-derived embryonic tissues. The present study was done to determine at what

stage cyclophosphamide first affects embryonic development and whether it selectively affects inner cell mass cells. The results show that there is a clear deficit in cell number as early as day 3 of gestation in embryos sired by cyclophosphamide-treated males. This deficit is apparently non-selective, affecting both the inner cell mass and trophectoderm populations, and is characterized by decreased DNA synthetic ability.

## Materials and Methods

### Animals

Adult male (300-315 g) and virgin female (225-250 g) Sprague-Dawley rats were obtained from Charles River Canada Inc. (St. Constant, Québec, Canada) and housed in the McIntyre Animal Centre. Food and water were provided *ad libitum* and animals were maintained on a 14-hr L:10-hr D cycle.

### Treatment and Mating

Male rats were randomly divided into two groups (control and cyclophosphamide-treated). Males were gavaged as previously described (20). Treated males received 6.0 mg/kg/day cyclophosphamide (Procytox$^{R}$, Frank Horner, Montréal, Québec, Canada) and control males received an equal volume of saline. Males were bred to virgin female rats in proestrus during the fifth week of treatment. Males were not gavaged on the day of their overnight mating, to

avoid seminal transmission of cyclophosphamide (28). The morning after mating (designated day 0 of gestation), females were checked for spermatozoa in the vaginal smear. Sperm-positive females were killed on day 2 (8-9 am), day 2.5 (8-9 pm), day 3 (10-11 am), day 4 (10-11 am), and day 4.5 (6:30 - 7:30 pm) of gestation. Embryos were collected from oviducts or uteri and photographed under the light microscope (Wild Leitz, Montréal, Québec, Canada).

Nuclear Staining

For cell number assessment on day 2, 61 embryos sired by 8 control males and 29 embryos sired by 6 treated males were used. On day 2.5, 63 embryos sired by 8 control males and 72 embryos sired by 8 treated males were assessed. On day 3.0, 58 embryos sired by 9 control males and 48 embryos sired by 10 males were assessed. On day 4, 39 embryos sired by 8 control males and 30 embryos sired by 6 treated males were assessed. Embryos were flushed from oviducts or uteri in modified M2 medium (mM2, without phenol red), rinsed in mM2, and incubated in modified M16 medium (mM16, without phenol red) containing 50 μg/ml Hoechst 33258 (Molecular Probes, Eugene, Oregon, U.S.A.) at 37°C for 20 minutes, then washed three times in Earl's Balanced Salt Solution (EBSS; GIBCO, Burlington, Ontario, Canada) containing 10% fetal calf serum and penicillin/streptomycin (EBSS/FCS). Embryos were then washed in two drops of distilled water and placed in a small drop of mounting medium. Nuclei were

photographed and counted using fluorescence microscopy (A filter, Wild-Leitz).

Thymidine Uptake

Thymidine uptake was assessed using the method described by Heyner et al. (29). For day 3 of gestation, 60 embryos sired by 6 control males and 68 embryos sired by 6 treated males were used; for day 4 of gestation, 61 embryos sired by 6 control males and 61 embryos sired by 5 treated males were used. Embryos were flushed in mM2 from oviducts or uteri of females on day 3 (am) or day 4 (am) of gestation, equilibrated in mM16 at 37°C and 5% $CO_2$ / 95% air for 1 hour, then transferred to a 40 µl drop of mM16 containing 50 µCi/ml [$^3$H]-thymidine (ICN Bionuclear, Montréal, Québec, Canada) under paraffin oil. After 26.5 hours incubation at 37°C under 5% $CO_2$ / 95% air, embryos were washed in ice cold mM2 containing 50 µg/ml unlabelled thymidine (Sigma Chemical Company, St. Louis, MO) and placed in an Eppendorf tube containing 25 µl mM2 with 2% BSA. After the addition of 25 µl of 10% trichloroacetic acid (TCA), the sample was incubated on ice for 15-20 minutes, then spun in a microcentrifuge for 5 minutes at 4°C. The pellet was washed twice with 100 µl cold 10% TCA, and the supernatants were combined. The pellet was dissolved with 30 µl of 1 N NaOH, and the pH was lowered with 100 µl of 1 N HCl. The pellet and supernatant fractions were placed in scintillation vials with 10 ml Scintiverse E (Fisher

Scientific, Montréal, Québec, Canada) and counted in an LKB 1217 Rackbeta Liquid Scintillation Counter. Appropriate blanks were subtracted from all values which were converted to disintegrations per minute by the necessary quench corrections. Disintegrations per minute were normalized for the number of embryos in each sample, to render a value in dpm/embryo. This value was further normalized for cell number (obtained in the cell counting experiment) to render a value in dpm/cell. In order to confirm that the amount of [$^3$H]-thymidine in the TCA-soluble fraction was indeed a measure of DNA synthesis, control embryos were cultured for 24 hours in medium containing 50 $\mu$Ci/ml [$^3$H]-thymidine in the presence or absence of the DNA synthesis inhibitor aphidicolin (Sigma Chemical Company, St. Louis, MO) to determine background incorporation of label. In the presence of 2.5 $\mu$g/ml aphidicolin, a concentration which blocks 90% of DNA synthesis (30), incorporation of [$^3$H]-thymidine into the TCA-soluble fraction was decreased 92% (4075 dpm/embryo without aphidicolin; 334 dpm/embryo with aphidicolin). Thus incorporation of radiolabel into the TCA-soluble fraction can be considered an accurate measure of DNA synthesis.

Chromosome Preparations

The number of chromosomes in day 4 (am) embryos was assessed using the method of Garside and Hillman (31). Embryos were collected from pregnant mothers on day 4 of

gestation by uterine flushing with alpha-Minimum Essential Medium (α-MEM, GIBCO, Burlington, Ontario, Canada) containing 10% fetal calf serum and penicillin/streptomycin (α-MEM/FCS). Isolated embryos were rinsed, and placed in α-MEM containing 1 μg/ml colcemid (Sigma Chemical Company, St. Louis, MO) for an hour at 37°C in 5% $CO_2$ / 95% air. Embryos were transferred to a hypotonic 1% sodium citrate solution for 3 minutes at room temperature. Embryos were then placed in individual wells of a four-well plate containing fixative (3:1, methanol:glacial acetic acid) precooled to -20°C, and incubated at 4°C for 10 minutes. Each embryo was placed in the centre of a microscope slide in the smallest volume of fixative possible. A drop of glacial acetic acid:0.01% EDTA in distilled $H_2O$ (3:1) was deposited onto the embryo to dissociate the cells. This was followed by three successive drops of 100% methanol:$H_2O$:glacial acetic acid (9:4:3). Each drop was added ten seconds apart to allow for the spreading of the nuclei on the slide. The embryonic nuclei were then fixed onto the slide using a final drop of fixative (3:1, methanol:glacial acetic acid). Slides were dried on a 37°C heating plate, stained with Giemsa (Gurr, BDH, Montréal, Québec, Canada), and examined by light microscopy. Slides were coded for blinded assessment of chromosome number, and counted twice.

Differential Staining

Blastocysts obtained on the evening of day 4 (day 4.5)

were differentially stained to count the number of cells in the inner cell mass and the trophectoderm lineages. For the control group, 43 embryos sired by 8 control males were assessed. For the treated group, 40 embryos sired by 8 treated males were assessed. Differential staining was done as described by Pampfer et al. (32). Blastocysts were treated for 5 minutes at 37°C in a solution of 0.4% pronase (Protease Type XIV, Pronase E; Sigma Chemical Company, St. Louis, MO) in EBSS to remove the zonae. They were washed twice in EBSS/FCS, before partial immunolysis of the trophectoderm cells. Immunolysis was done by first incubating the blastocysts for 30 minutes at 37°C in rabbit anti-rat antibody (33) diluted 1:6.5 in EBSS. Because of tight junctions between trophectoderm cells, the antibody was not able to bind to the inner cell mass cells. Blastocysts were then washed three times in EBSS. Trophectoderm cells were partially lysed with 10% guinea pig complement (GIBCO, Burlington, Ontario, Canada) in EBSS which also contained 20 μg/ml Hoechst 33258 and 60 μg/ml propidium iodide (both from Molecular Probes, Eugene, Oregon, USA). Blastocysts were incubated in this complement solution for 10 minutes at 37°C, then washed three times in EBSS/FCS before mounting on a microscope slide. Both Hoechst 33258 and propidium iodide are chromatin dyes, but propidium iodide does not pass through cell membranes. Thus propidium iodide entered only the trophectoderm cells which

had been permeabilized by the antibody and complement. Hoechst 33258 is able to freely cross membranes and therefore was able to stain the nuclei of both inner cell mass cells and trophectoderm cells. Thus inner cell mass cell nuclei appeared blue, having been stained only with Hoechst 33258, whereas the trophectoderm cell nuclei, which were stained with both Hoechst 33258 and propidium iodide, appeared pink. Differentially stained blastocysts were assessed by fluorescence microscopy using an A filter to count the number of nuclei in the two cell lineages.

Statistical Analysis

All data were analyzed on a per male basis by one-way analysis of variance (ANOVA), chi-square analysis or simple regression analysis using the Complete Statistical System (CSS) software program (Statsoft Inc., Tulsa, OK). The level of significance was taken as $P<0.05$.

## Results

Cell Number

Randomly selected embryos from each male were analyzed at different gestational ages by nuclear Hoechst 33258 staining. There was no difference in the number of cells on day 2 or day 2.5 of gestation between embryos sired by cyclophosphamide-treated males and control males (Fig. 1). Day 3 embryos sired by cyclophosphamide-treated males had a significant 37% reduction in cell number compared to those

sired by control males (Fig. 1). The decrease in cell number seen on day 3 reached more than 50% by day 4 of gestation (Fig. 1).

In order to determine the uniformity of cyclophosphamide effects, the distribution of embryonic cell numbers was examined (Fig. 2). The cell numbers in embryos sired by drug-treated males were distributed normally; this indicates a homogeneous deleterious effect of paternal cyclophosphamide treatment. On day 2 of gestation the majority of embryos in both paternal treatment groups (control 77%; treated 62%) were at the 2-cell stage (Fig. 2). Twelve hours later the majority (control 57%; treated 64%) had advanced to the 4-cell stage (Fig. 2). By day 3, the median number of cells in the control group was 8-9, while in the treated group, similar numbers of embryos could be found with 4-5 cells and 8-9 cells, suggesting that cell division was becoming increasingly asynchronous (Fig. 2). By day 4, a clear dissociation of the distribution pattern for control and treated groups was noticed, with the median for control being at 30-35 cells and for treated at 12-17 cells (Fig. 2).

The increase in cell number between days 3 and 4 and the presence of mitotic figures in several of the embryos sired by cyclophosphamide-treated males indicated that at least some cell proliferation was occurring. In order to determine the cell doubling time in the two treatment

groups, the cell numbers were converted to natural logarithms and expressed as a function of time after fertilization (Fig. 3). Using regression line analysis, the mean cell doubling time of control embryos between 54 and 104 hours after fertilization was found to be 12 hours. However, the mean cell doubling time for embryos sired by cyclophosphamide-treated males was 16 hours. Thus paternal cyclophosphamide treatment caused a reduction in the proliferation of early embryonic cells.

DNA Synthesis and Chromosome Complement

The cell proliferation of embryos sired by control and drug-treated males was further investigated by measuring the relative uptake of tritiated thymidine into DNA. Two windows of early development were chosen for analysis of DNA synthesis: day 3.0 to day 4.0, and day 4.0 to day 5.0. The incorporation of [$^3$H]-thymidine was dramatically lower in embryos sired by cyclophosphamide-treated males after culture on both day 3 and day 4 of gestation, when compared to controls (Fig. 4a). Surprisingly, the relative [$^3$H]-thymidine incorporation of embryos sired by cyclophosphamide-treated males compared to controls was in fact much lower when cultured from day 3 (18%) than from day 4 (34%); this suggests that in embryos sired by treated males, the rate of DNA synthesis is more affected in the day 3 to day 4 window than in the day 4 to day 5 window. The

decrease in the number of cells on day 3 and day 4 in the embryos sired by treated males does not fully account for the decrease in [$^3$H]-thymidine incorporation (Fig. 4b). It thus appears that even in those cells present, the DNA synthesis is decreased in embryos sired by treated males when compared to controls.

Because of the similarity with respect to decreased proliferation rate between the pre-implantation development of embryos sired by cyclophosphamide-treated males and that of haploid gynogenetic mouse embryos (34), chromosome numbers in day 4 embryos were assessed. Paternal chromosomes were present in embryos sired by cyclophosphamide-treated males (Fig. 5). For the control group, 60 embryos were examined, yielding 26 analyzable spreads from the progeny of 6 different control males. For the treated group, 79 embryos were examined, yielding 19 analyzable spreads from the progeny of 7 different treated males. The proportion of analyzable spreads for the two groups (control 43%; treated 24%) was significantly different by Chi square analysis (P=.026). This may be simply due to the presence of fewer cells in the treated group, or may represent decreased mitotic activity. Chromosomes in analyzable spreads which were inherited from treated males were apparently able to condense and decondense in response to cellular mitotic control mechanisms and had no gross cytogenetic abnormalities (Fig.

5). Where multiple analyzable spreads were available for a single embryo, the mean chromosome number was calculated. In turn, for each male a mean chromosome number was calculated from the embryos analyzed. The quality of the chromosome spreads was such that some chromosome loss occurred, but to an equal extent in both groups. The mean embryonic chromosome number for control males ranged from 34.7 to 42.0, with an overall mean of 39.5 ± 1.1. The mean embryonic chromosome number for cyclophosphamide-treated males ranged from 36.5 to 42.0, with an overall mean of 39.9 ± 0.7. These values are not significantly different from one another (P=.783). There were clearly embryos with a full diploid complement of 42 chromosomes in both control and treated groups. Although the possible selection of the small percentage of healthy embryos in the litters sired by treated males cannot be ruled out, these results indicate that the failure of these embryos is seemingly not due simply to a lack of paternal chromosomes, but to some more subtle effect.

Lineage Specificity

The apparently selective loss of inner cell mass-derived embryonic tissue in the day 7 implantation site (27) suggested that the population of cells which were dying in embryos sired by cyclophosphamide-treated males was the inner cell mass cells. To determine whether the loss of embryonic cells was lineage-specific, blastocysts from

control and cyclophosphamide-treated males were differentially stained for the inner cell mass and trophectoderm cell lineages.

Day 4.5 blastocysts sired by cyclophosphamide-treated males were visibly retarded when compared to those sired by control males (Fig. 6a,b). Most of the embryos sired by control males formed a large blastocoel cavity, with a well-defined inner cell mass at one pole (Fig. 6a). In contrast, only a few of the embryos sired by treated males had even begun to cavitate and many had not achieved the morula stage (Fig. 6b). In many embryos sired by cyclophosphamide-treated males there were blastomeres which were abnormal in both size and shape (Fig. 6b). After the differential staining protocol, the blastocysts sired by treated males (Fig 6d) had considerably fewer pink- and blue-stained nuclei than those sired by control males (Fig. 6c). Presumably tight junctions were able to form permeability seals in the embryos sired by treated males, since blue cell nuclei were present, indicating that the inner cells were protected from antibody binding.

The total number of stained nuclei in embryos sired by treated males was approximately 50% that in embryos sired by control males (Fig. 7) This is consistent with the effect of paternal treatment on cell number at day 4.0. The deleterious effect of paternal cyclophosphamide treatment at day 4.5 was not lineage-specific; the cell numbers of both

the inner cell mass and the trophectoderm were decreased by approximately 50% (Fig. 7).

Thus there was a significant decrease in cell number in embryos sired by drug-treated males as early as day 3 of gestation, which by day 4 reached half the control cell number, with proportional decreases in both the embryonic and the extra-embryonic cell lineages.

## Discussion

More than eighty percent of the embryos sired by males treated with chronic low doses of cyclophosphamide for four weeks die early after implantation (19,20). The present study shows that this loss is not a sudden failure, but a slow, gradual loss of embryonic cells or a retardation of their growth, detectable by the third day of gestation. This depletion of cells does not prevent the embryos sired by treated males from implanting into the uterine wall (27). Clearly the cells in the embryos are still dividing because there is an increase in number from gestational day 3 to day 4, tritiated thymidine is incorporated into DNA, and chromosomes can be isolated in metaphase. It is still unresolved whether all of the cells are dividing more slowly or some cells are dying while others still proliferate.

In the pre-implantation development of the mouse, the cell cycle length becomes increasingly shorter as the embryo progresses through the cleavage stages to gastrulation (35);

similar data for the rat are not available. Although it is possible that the day 3 to day 5 window may be a selection point for cells able to undergo DNA synthesis, it is more likely that the length of the cell cycle in the more advanced embryos sired by control males is shorter than that in the retarded embryos sired by treated males; thus there is more DNA synthesis and hence more uptake of radiolabel in the controls.

One recent mouse chimera study, using parthenogenetic cells, suggested that the absence of paternal chromosomes is largely incompatible with the maintenance of specific differentiated cell types, and that paternal chromosomes are important for the regulation of proliferation of all cell types (36). The cell doubling time obtained in the present study for rat embryos sired by cyclophosphamide-treated males is very similar to that of haploid gynogenetic mouse embryos completely lacking in paternal chromosomes (34). It is clear, however, from the present study that embryos sired by treated males are not haploid.

The present study also shows that the decrease in cell number in the embryos sired by cyclophosphamide-treated males is not lineage-specific at the blastocyst stage, despite the apparent specificity of inner cell mass-derived tissue loss in the day 7 implantation site (27). The fact that both the inner cell mass and the trophoblast populations are proportionally decreased in the blastocyst

indicates that the embryonic and extra-embryonic cell lineages are not equally able to handle a drastic depletion in their population. There is some evidence in the mouse for differential sensitivities of the inner cell mass cells and trophectoderm to direct exposure to various agents, most likely due to an increased rate of proliferation in the inner cell mass (37,38,32). In the mouse blastocyst, the inner cell mass is more sensitive than the trophectoderm to the direct action of a variety of agents which include x-irradiation (39), [$^{3}$H]-thymidine (long-term exposure)(40), mitomycin C (41), aphidicolin (30), and cyclophosphamide (42,43). This differential sensitivity between inner cell mass and trophectoderm cells may in part be due to a greater ability of the trophectoderm cells to repair DNA damage. Pedersen and Cleaver (44) showed that there was a greater degree of unscheduled DNA synthesis in trophectoderm than in inner cell mass in mouse blastocysts after UV irradiation.

The activation of the mouse embryonic genome occurs at the 2-cell stage, and progressively more embryonic genes are thought to be turned on as embryogenesis progresses and as maternal mRNA is degraded (45-47). In the mouse it appears that the paternal genome is not required until the 8-cell stage for normal development to term (48). At the 8- to 16-cell stage, rat embryonic cells begin to differentiate into the inner cell mass cells and the trophectoderm cells. This differentiation is dependent on embryonic transcripts coding

for growth factors and other important functional proteins (46).

Although nuclear transfer experiments in mouse zygotes have led to the concept of paternal genes being important for the development of extra-embryonic, rather than embryonic, tissues in early post-implantation development (reviewed in 49), it has been shown that paternal DNA strands segregate randomly into both the inner cell mass and trophectoderm (50). Although the inner cell mass cells are considered to be the "stem cells" of the early embryo (51), studies by microinjecton of the origin of the inner cell mass showed that in mouse embryos the outer cells can have inner descendants until the late morula/early blastocyst stage (52). Thus, in embryos sired by cyclophosphamide-treated males, if the inner cell mass cell population is depleted and the trophectoderm cells are less affected, trophectoderm cells may still be able to proliferate, incorporating [$^3$H]-thymidine, and provide compensatory inner cell mass cells.

In the mouse, autosomal monosomy results in lethality in the pre- or peri-implantation period (reviewed in 53), indicating that all paternal autosomes carry genes important for embryo development. Cyclophosphamide somehow alters the paternal contribution to the embryo and causes abnormal pre-implantation development, most likely targeting the DNA and functionally eliminating paternal genes essential for early

embryo growth. It is also possible that the DNA synthesis in embryos sired by drug-treated males is impeded by physical changes in paternal chromosomal structure or arrangement in the nucleus as a result of cyclophosphamide exposure.

Chronic cyclophosphamide exposure interferes with spermatozoal decondensation (54), a measure of spermatozoal function. The chromatin of the spermatozoon decondenses in the fertilized ovum from a tightly-packed inactive DNA conformation to an active dispersed conformation. Spermatozoal decondensation can be studied in vitro in the presence of a disulfide reducing agent (55). The in vitro decondensation of spermatozoa is affected by 6-weeks exposure to cyclophosphamide. In the spermatozoa of treated males, the chromatin is unable to disperse in the second phase of decondensation in vitro, leading to the speculation that cyclophosphamide may cross-link the histone-like protamines (54).

Dean and Rossant (30) obtained results strikingly similar to those in the present model in mouse embryos treated with aphidicolin. The inhibition of DNA synthesis resulted in embryos containing half the normal number of cells. Blastocyst formation was not affected in aphidicolin-treated embryos despite the paucity of cells, and most resulting implantation sites contained only trophoblast giant cells. DNA synthesis was inhibited to a

greater degree, however, in the aphidicolin-treated mouse embryos than was found in the embryos sired by cyclophosphamide-treated males in the present study. Until cause and effect can be clarified, the question of whether the mechanism of paternal cyclophosphamide embryotoxicity is genetic and/or epigenetic in nature will remain unanswered.

Cyclophosphamide damage to the paternal genome can lethally disrupt embryogenesis even from its very early stages, dramatically reducing proliferation and consequently cell number, leading to embryo death. This provides an excellent experimental model for understanding the role of the paternal genome in early embryo development.

Acknowledgements

The authors wish to acknowledge the generous provision of rabbit anti-rat antibody by Dr. J. Carnegie, Loeb Institute, Ottawa Civic Hospital, Ottawa, Canada.

References


1. Fairley KF, Barrie JU, Johnson W. Sterility and testicular atrophy related to cyclophosphamide therapy. Lancet 1972; 1:568-569.
2. Qureshi MSA, Pennington JH, Goldsmith HJ, Cox PE. Cyclophosphamide therapy and sterility. Lancet 1972; 2:1290-1291.
3. Fukutani K, Ishida H, Shinohara M, Minowada S, Niijima T, Hijikata K, Izawa Y. Suppression of spermatogenesis in patients with Behçet's disease treated with cyclophosphamide and colchicine. Fertil Steril 1981; 36:76-80.
4. Fritz H, Müller D, Hess R. Embryolethality in the mouse following treatment of males with cyclophosphamide at specific germ cell stages. Agents Actions 1973; 3:35-37.
5. Rathenberg R, Müller D. Comparative cytogenetic studies of the influence of phenylbutazone and cyclophosphamide on spermatogenesis in the mouse. Agents Actions 1972; 2:180-185.
6. Sotomayor RE, Cumming RB. Induction of translocations by cyclophosphamide in different germ cell stages of male mice: cytological characterization and transmission. Mutat Res 1975; 27:375-388.

7. Velez de la Calle JF, de Queiroz F, Garnier DH, Kercret H, Folliot R, Jégou B. Reproductive effects of the anticancer drug cyclophosphamide in male rats at different ages. Arch Androl 1989; 22:251-263.

8. Jenkinson PC, Anderson D. Malformed foetuses and karyotype abnormalities in the offspring of cyclophosphamide and allyl alcohol-treated male rats. Mutat Res 1990; 229:173-184.

9. Abraham SK, Fränz J. Induction of sister-chromatid exchanges by chemotherapeutic drugs in spermatogonia of mice: effects of procarbazine, adriamycin, cyclophosphamide and mitomycin C. Mutat Res 1983; 108:373-381.

10. Lahdetie J. Induction and survival of micronuclei in rat spermatids. Comparison of two meiotic micronucleus techniques using cyclophosphamide. Mutat Res 1988; 203:47-53.

11. Moreland FM, Sheu CW, Springer JA, Green S. Effects of prolonged chemical treatment with cyclophosphamide and 6-mercaptopurine in the dominant lethal test system. Mutat Res 1981; 90:193-199.

12. Hales BF, Crosman K, Robaire B. Increased postimplantation loss and malformations among the $F_2$ progeny of male rats chronically treated with cyclophosphamide. Teratology 1992; 45:671-678.

13. Adams PM, Fabricant JD, Legator MS. Cyclophosphamide-induced spermatogenic effects detected in the $F_1$ generation by behavioral testing. Science 1981; 211:80-82.

14. Adams PM, Fabricant JD, Legator MS. Active avoidance behavior in the $F_1$ progeny of male rats exposed to cyclophosphamide prior to fertilization. Neurobehav Toxicol Teratol 1982; 4:531-534.

15. Fabricant JD, Legator MS, Adams PM. Post-meiotic cell mediation of behavior in progeny of males rats treated with cyclophosphamide. Mutat Res 1983; 119:185-190.

16. Dulioust EM, Nawar NY, Yacoub SG, Ebel AB, Kempf EH, Auroux MR. Cyclophosphamide in the male rat: new pattern of anomalies in the third generation. J Androl 1989; 10:296-303.

17. Auroux MR, Dulioust EM, Nawar NY, Yacoub SG. Antimitotic drugs (cyclophosphamide and vinblastine) in the male rat: deaths and behavioral abnormalities in the offspring. J Androl 1986; 7:378-386.

18. Auroux MR, Dulioust EJB, Nawar NNY, Yacoub SG, Mayaux MJ, Schwartz D, David G. Antimitotic drugs in the male rat: behavioral abnormalities in the second generation. J Androl 1988; 9:153-159.

19. Trasler JM, Hales BF, Robaire B. Paternal cyclophosphamide treatment of rats causes fetal loss and malformations without affecting male fertility. Nature 1985; 316:144-146.

20. Trasler JM, Hales BF, Robaire B. Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. Biol Reprod 1986; 34:275-283.

21. Trasler JM, Hales BF, Robaire B. A time-course study of chronic paternal cyclophosphamide treatment in rats: effects on pregnancy outcome and the male reproductive and hematologic systems. Biol Reprod 1987; 37:317-326.

22. Hales BF, Robaire B. Reversibility of the effects of chronic paternal exposure to cyclophosphamide on pregnancy outcome in rats. Mutat Res 1992; 229:129-134.

23. Rossant J. Development of extraembryonic cell lineages in the mouse embryo. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian Embryonic Development. New York: Cambridge University Press; 1986: 97-120.

24. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 1984; 37:179-183.

25. Barton SC, Surani MAH, Norris ML. Role of

paternal and maternal genomes in mouse development. Nature 1984; 311:374-376.

26. Surani MAH, Barton SC, Norris ML. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature 1984; 308:548-550.

27. Kelly SM, Robaire B, Hales BF. Paternal cyclophosphamide treatment causes postimplantation loss via inner cell mass-specific cell death. Teratology 1992; 45:313-318.

28. Hales BF, Smith S, Robaire B. Cyclophosphamide in the seminal fluid of treated males: transmission to females by mating and effect on pregnancy outcome. Toxicol Appl Pharmacol 1986; 84:423-430.

29. Heyner S, Rao LV, Jarett L, Smith RM. Preimplantation mouse embryos internalize maternal insulin via receptor-mediated endocytosis: pattern of uptake and functional correlations. Dev Biol 1989; 134:48-58.

30. Dean WL, Rossant J. Effect of delaying DNA replication on blastocyst formation in the mouse. Differentiation 1984; 26:134-137.

31. Garside W, Hillman N. A method for karyotyping mouse blastocyst embryos developing from in vivo and in vitro fertilized eggs. Experientia 1985; 41:1183-1184.

32. Pampfer S, Vanderheyden I, Michiels B, De Hertogh R. Cell allocation to the inner cell mass and the trophectoderm in rat embryos during in vivo preimplantation development. Roux's Arch Dev Biol 1990; 198:257-263.

33. Carnegie JA, Cabaca O. The influence of extracellular matrix components on the proliferation and migration of inner cell mass-derived parietal endodermal cells. Biol Reprod 1991; 45:572-580.

34. Henery CC, Kaufman MH. Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. Mol Reprod Dev 1992; 31:258-263.

35. Pedersen RA. Potency, lineage, and allocation in preimplantation mouse embryos. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian Embryonic Development. New York: Cambridge University Press; 1986: 3-33.

36. Fundele RH, Norris ML, Barton SC, Fehlau M, Howlett SK, Mills WE, Surani MA. Temporal and spatial selection against parthenogenetic cells during development of fetal chimeras. Development 1990; 108:203-211.

37. Barlow P, Owen DAJ, Graham C. DNA synthesis in the preimplantation mouse embryo. J Embryol Exp

Morph 1972; 27:431-445.

38. Handyside AH, Hunter S. Cell division and death in the mouse blastocyst before implantation. Roux's Arch Dev Biol 1986; 195:519-526.

39. Goldstein LS, Spindle AI, Pedersen RA. X-Ray sensitivity of the preimplantation mouse embryo in vitro. Radiat Res 1975; 62:276-287.

40. Snow MHL. Abnormal development of pre-implantation mouse embryos grown in vitro with [$^{3}$H]-thymidine. J Embryol exp Morph 1973; 29:601-615.

41. Tam PPL. Postimplantation development of mitomycin C-treated mouse blastocysts. Teratology 1988; 37:205-212.

42. Spielmann H, Jacob-Müller U, Eibs H-G, Beckford W. Investigations on cyclophosphamide treatment during the preimplantation period. I. Differential sensitivity of preimplantation mouse embryos to maternal cyclophosphamide treatment. Teratology 1981; 23:1-5.

43. Kola I, Folb PI, Parker MI. Maternal administration of cyclophosphamide induces chromosomal aberrations and inhibits cell number, histone synthesis, and DNA synthesis in preimplantation mouse embryos. Teratogenesis Carcinog Mutagen 1986; 6:115-127.

44. Pedersen RA, Cleaver JE. Repair of UV damage to DNA of implantation-stage mouse embryos in vitro. Exp Cell Res 1975; 95:247-253.

45. Schultz GA. Utilization of genetic information in the preimplantation mouse embryo. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian Embryonic Development. New York: Cambridge University Press; 1986:239-265.

46. Schultz GA, Heyner, S. Gene expression in pre-implantation mammalian embryos. Mutat Res 1992; 296:17-31.

47. Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 1990; 26:90-100.

48. Renard J-P, Babinet C, Barra J. Participation of the paternal genome is not required before the eight-cell stage for full-term development of mouse embryos. Dev Biol 1991; 143:199-202.

49. Surani MAH. Evidence and consequences of differences between maternal and paternal genomes during embryogenesis in the mouse. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian Embryonic Development. New York: Cambridge University Press; 1986: 401-435.

50. Ito K, McGhee JD, Schultz GA. Paternal DNA

strands segregate to both trophectoderm and inner cell mass of the developing mouse embryo. Genes Dev 1988; 2:929-936.

51. Winkel GK, Pedersen RA. Fate of the inner cell mass in mouse embryos as studied by microinjection of lineage tracers. Dev Biol 1988; 127:143-156.

52. Pedersen RA, Wu K, Balakier H. Origin of the inner cell mass in mouse embryos: cell lineage analysis by microinjection. Dev Biol 1986; 117:581-595.

53. Magnuson T, Epstein CJ. Genetic expression during early mouse development. In: Bavister BD (ed.), The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation *in Vitro*. New York: Plenum Press; 1987: 133-150.

54. Hales BF, Robaire B. Paternally mediated effects on progeny outcome. In: Whitcomb RW, Zirkin BR (eds.), Understanding Male Infertility: Basic and clinical approaches. Serono Symposia Publications, vol 98. New York: Raven Press; 1993: 307-320.

55. Zirkin BR, Chang TSK. Involvement of endogenous proteolytic activity in thiol-induced release of DNA template restrictions in rabbit sperm nuclei. Biol Reprod 1977; 17:131-137.

Figure 1

Effects of a 4 week treatment of male rats with cyclophosphamide on the number of cells in the embryo. The number of cells in embryos sired by control males (open bars) or by drug-treated males (bars with diagonal lines) is expressed as a function of the day of gestation. Paternal cyclophosphamide exposure had no effect on the number of cells in day 2 (P=0.699) or day 2.5 (P=0.495) embryos. Embryos sired by cyclophosphamide-treated males were significantly lower in cell number on day 3 (P=0.0029) and day 4 (P=0.000061) than embryos sired by control males.


40
30
20
10
0
Number of Cells
*
*
2.0
2.5
3.0
4.0
Gestational Age (in days)

Figure 2

The frequency of observation is expressed as a function of the number of cells in embryos sired by control (open bars) and cyclophosphamide-treated (bars with diagonal lines) males on day 2, day 2.5, day 3, and day 4 of gestation.

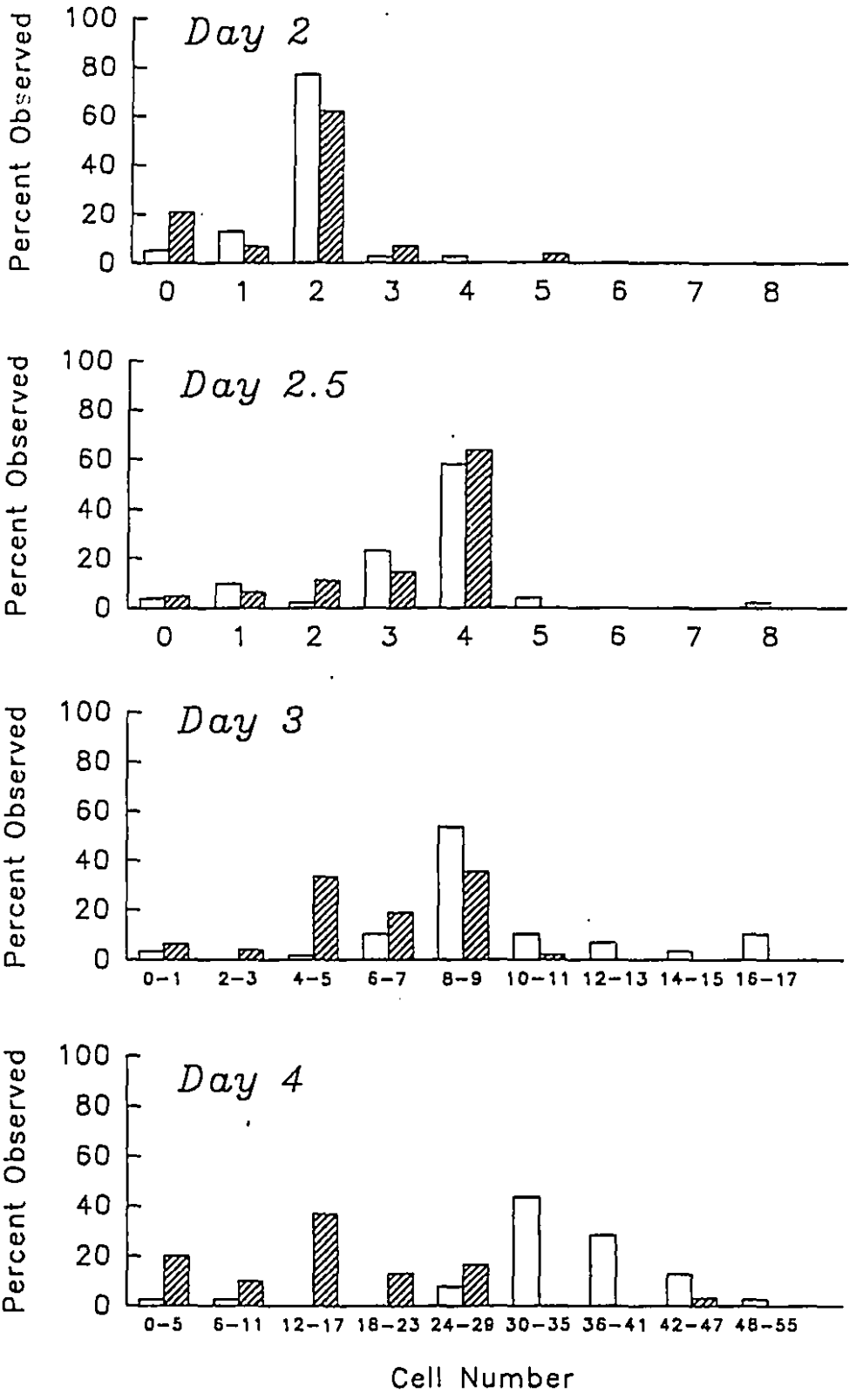

Day 2
Day 2.5
Day 3
Day 4
Percent Observed
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
0-1 2-3 4-5 6-7 8-9 10-11 12-13 14-15 16-17
0-5 6-11 12-17 18-23 24-29 30-35 36-41 42-47 48-55
Cell Number

Figure 3

Log of the total number of embryonic cells at various times during the preimplantation period, plotted against time after mating (mating time is considered to be 2 am in the morning of day 0) for embryos sired by control (open triangles) and cyclophosphamide-treated (filled triangles) males. For both groups regression lines are shown ($r = 1.00$ for both). The equation of the regression line for the control group was log(cell number)= $-2.439 + 0.058x + e$. The equation of the regression line for the cyclophosphamide-treated group was log(cell number)= $-1.686 + 0.043x + e$.

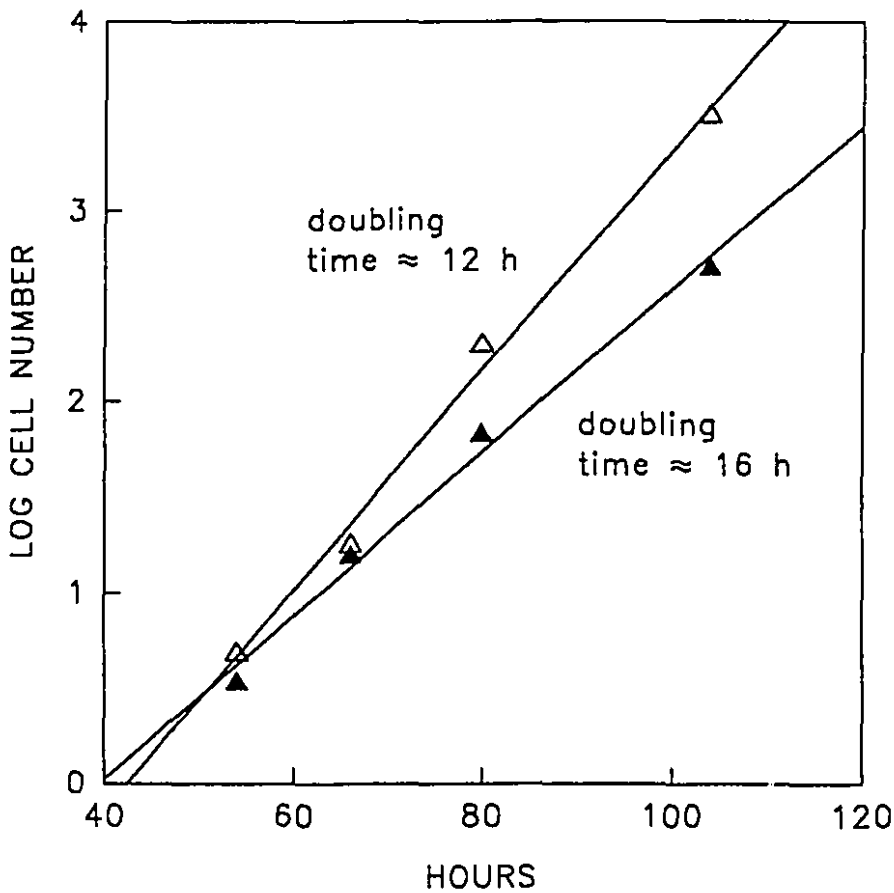

4
3
2
1
0
LOG CELL NUMBER
doubling
time ≈ 12 h
doubling
time ≈ 16 h
40
60
80
100
120
HOURS

Figure 4

Effects of 4-weeks of paternal cyclophosphamide treatment on [$^3$H]-thymidine incorporation in embryos cultured from day 3 to day 4 (3.0) and from day 4 to day 5 (4.0) of gestation. The [$^3$H]-thymidine incorporation is expressed as a function of gestational age at the start of the culture period. Values are expressed in (*a*) as incorporation per embryo, where in (*b*) they are expressed as incorporation per cell where the incorporation is corrected for the embryonic cell numbers obtained in the cell counting experiment (Figure 1). (*a*) Embryos sired by treated males (bars with diagonal lines) were significantly lower in [$^3$H]-thymidine incorporation in cultures begun on both day 3 (P=0.002) and day 4 (P=0.029) than embryos sired by control males (open bars). (*b*) After correcting for cell number, there is still a significant difference in cellular DNA synthesis on day 3 of gestation (P=.001), but not on day 4 of gestation (P=.545).

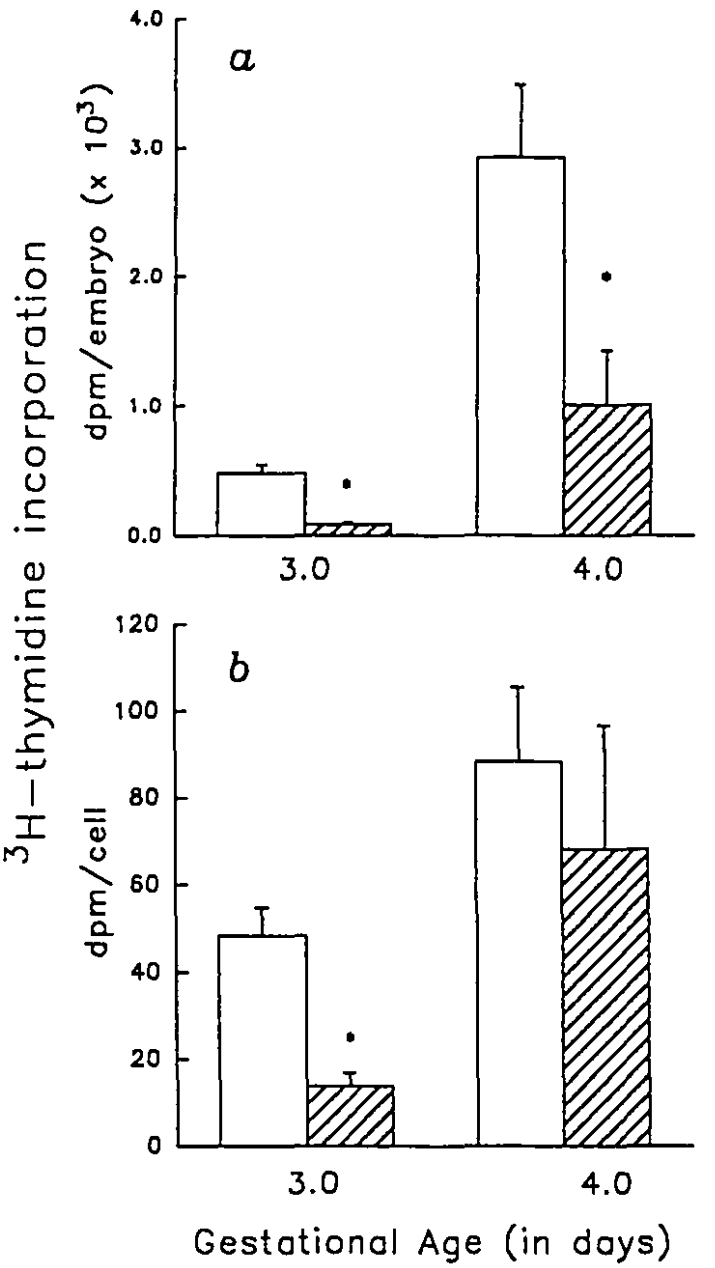

a
b
4.0
3.0
2.0
1.0
0.0
dpm/embryo (x 10³)
120
100
80
60
40
20
0
dpm/cell
3.0
4.0
3.0
4.0
³H–thymidine incorporation
Gestational Age (in days)

Figure 5

Light micrographs of metaphase chromosome spreads from day 4 embryos sired by control (a) and cyclophosphamide-treated (b) males. A full complement of 42 chromosomes is present in both cases. Bars represent 10 $\mu$m.

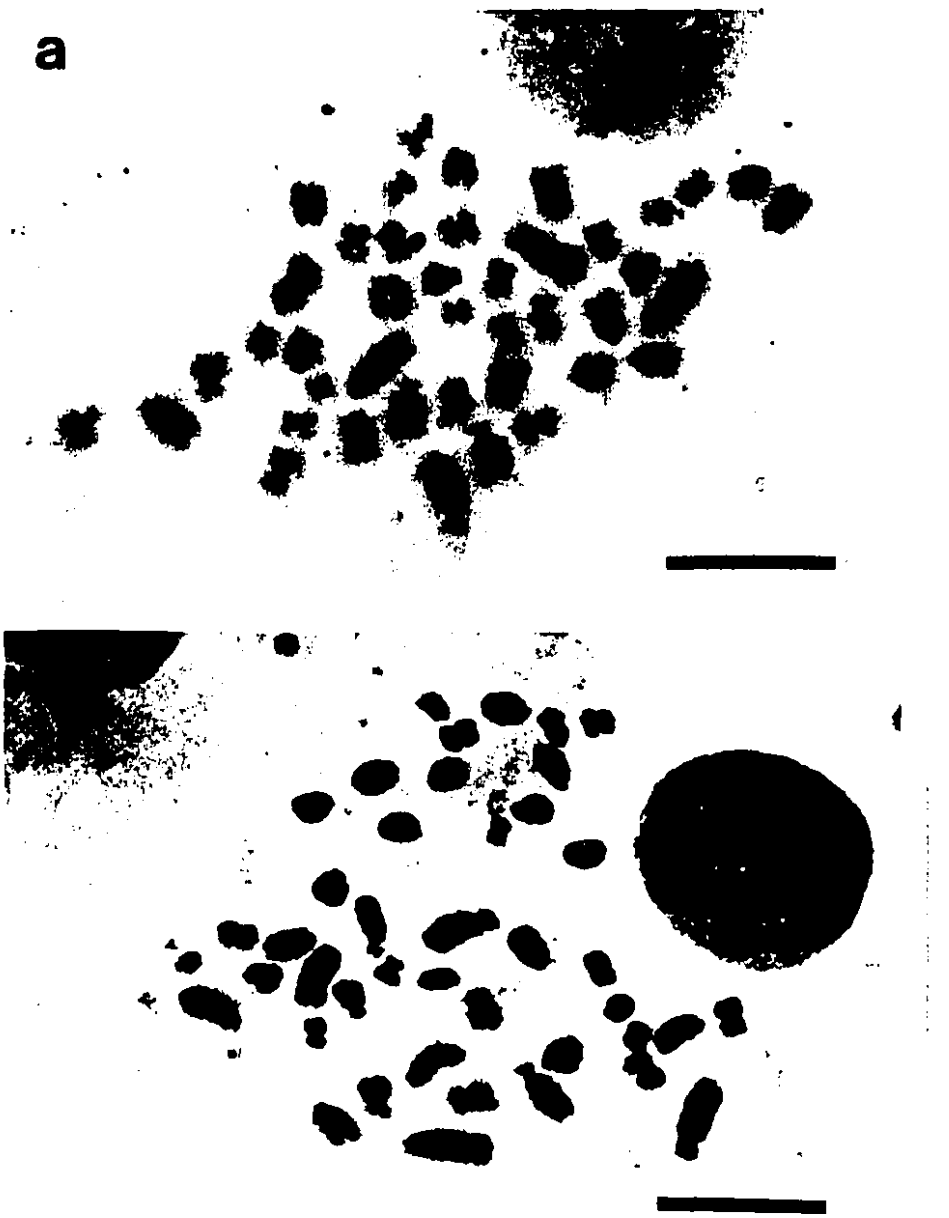

a

Figure 6

Light micrographs show representative day 4.5 blastocysts sired by control (a) and treated males (b). Fluorescence micrographs illustrate differential staining of day 4.5 blastocysts sired by control (c) and cyclophosphamide-treated (d) males. Inner cell mass cell nuclei are stained blue with Hoechst 33258, while trophectoderm cell nuclei are stained pink with both Hoechst 33258 and propidium iodide. Bars indicate 50 μm.

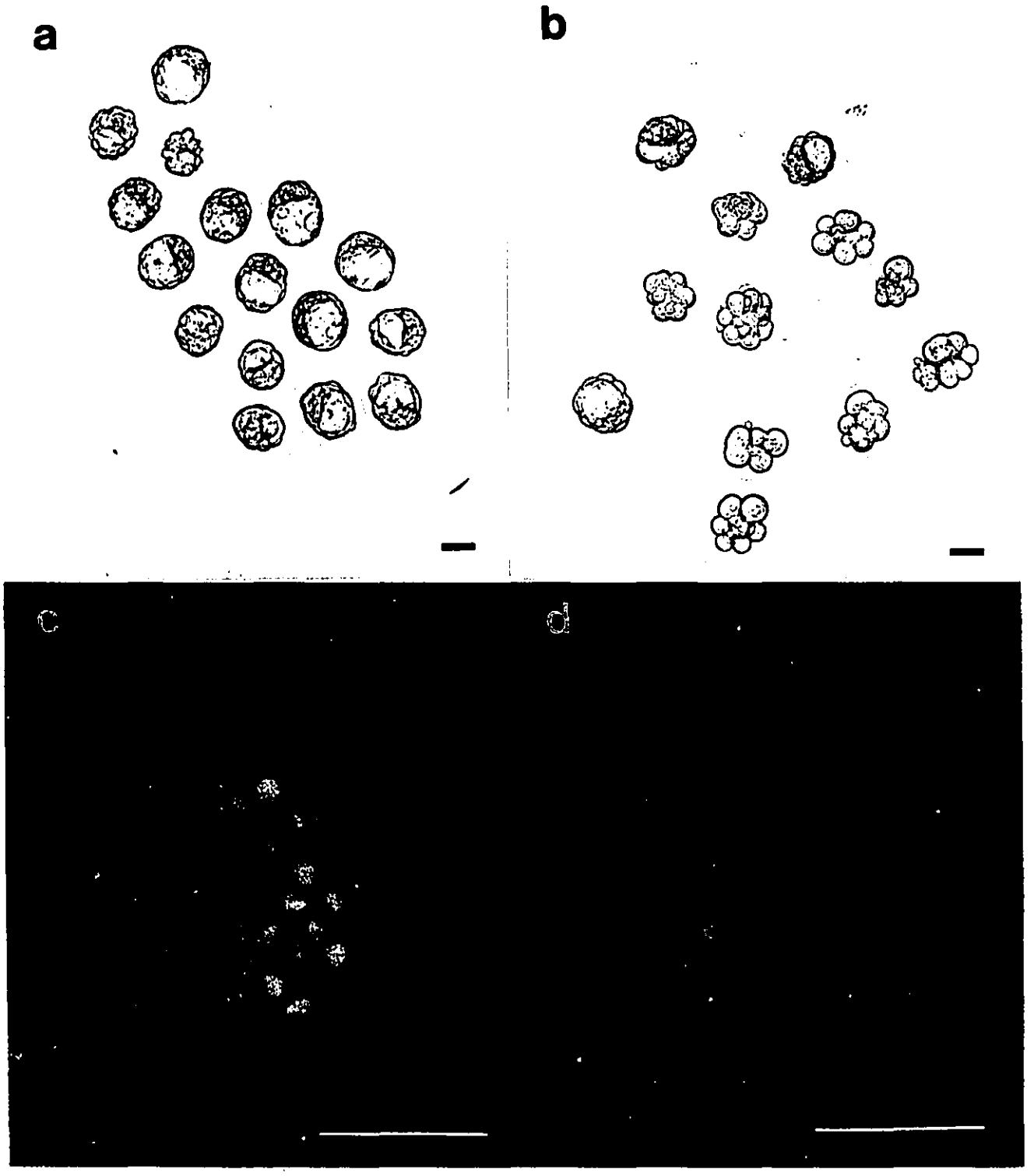
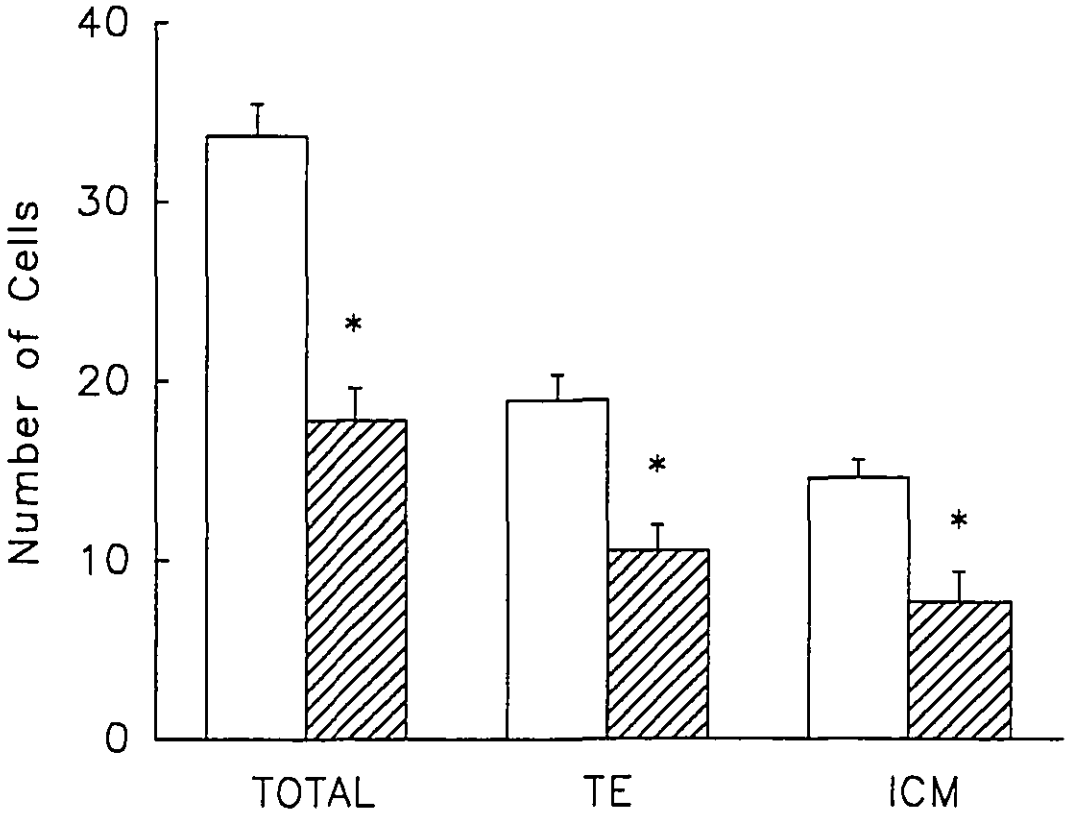

a
b
c
d

Figure 7

Effects of 4-weeks of paternal cyclophosphamide treatment on the day 4.5 embryo. The number of cells is expressed as a function of the cell lineage assessed. Embryos sired by treated males (bars with diagonal lines) were significantly lower (P=0.000013) in total number of cells (TOTAL) than embryos sired by control males (open bars). Paternal cyclophosphamide exposure caused a significant decrease in trophectoderm cells (P=0.000085) and inner cell mass cells (P=0.020). This decrease was proportional in both the trophectoderm (TE) and inner cell mass (ICM) cell lineages.


40
30
20
10
0
Number of Cells
*
*
*
TOTAL
TE
ICM

## Connecting Text - Chapter III to Chapter IV

The experiments in Chapter III demonstrated that paternal cyclophosphamide exposure resulted in a decrease in cell proliferation in embryos prior to implantation. DNA synthesis and cell number were significantly decreased in embryos sired by cyclophosphamide-treated males as early as day 3 of gestation. Moreover, this decrease in cell number was not specific to the inner cell mass lineage, but both the inner cell mass cells and the trophectoderm cells were proportionately decreased in number on day 4.5. In the next chapter, the ultrastructure of embryos sired by control and cyclophosphamide-treated males was compared at day 2.5, day 3 and day 4 of gestation, to determine 1) whether the decrease in embryonic cells was due to active cell death or to retardation of growth and 2) whether there is any gross alteration in the morphology of the cells or change in the progression of morphogenetic changes accompanying preimplantation development.

# Chapter IV

## Ultrastructure of Preimplantation Embryos Sired by Males Exposed to Chronic Low Doses of Cyclophosphamide

## Abstract

Cyclophosphamide is an anticancer drug which has reproductive consequences in both males and females. When male rats are exposed to chronic low doses of cyclophosphamide, there is a dramatic increase in the early postimplantation loss of their progeny. Embryonic development is first affected during the preimplantation stages, when the cells of embryos sired by treated males proliferate slowly; these embryos contain less than one-half the control cell number by day 4 of gestation. To determine whether the decrease in cell number is due to retarded development or active cell death, ultrastructural studies were done. Male Sprague-Dawley rats were treated orally with 6 mg/kg/day cyclophosphamide or an equivalent volume of saline (controls) for four weeks, then mated to virgin females. Embryos were collected on days 2.5, 3 and 4 of gestation, and prepared for electron microscopy. Some litter-specific inconsistency was observed in embryo morphology on day 2.5 and day 3.0 in both treatment groups. Embryos sired by treated males compacted between day 2.5 and day 3 despite their decreased cell numbers. By day 4 of gestation one third of embryos sired by treated males had not formed a trophectoderm ring, and showed signs of increased autophagy and cytoplasmic condensation. However, two thirds of the day 4 embryos in the treated group were

able to form a trophectoderm ring and appeared capable of response to differentiative signals. Thus it appears that the damage caused by paternal cyclophosphamide exposure has more effect on cell proliferation than on cell differentiation.

## Introduction

Ultrastructural studies have been key to understanding the progress of development in the preimplantation stages of embryo development. From the earliest studies of the ultrastructure of preimplantation rat embryos the polarization of cell organelles at the 8-cell stage has been described (Sotelo & Porter, '59; Izquierdo & Vial, '62). Ultrastructural studies have confirmed the delineation of cell lineages in the mouse blastocyst (Rossant et al., '83). Through careful studies of the dynamic changes in the cytology of the blastomeres, the mechanism and timing of rat blastocyst formation have been clarified (Schlafke & Enders, '67; Dvorak et al., '77). The segregation of cytokeratin sheets and the mitochondrial changes which accompany the maturation of rat blastocyst cells have been documented (Sotelo & Porter, '59; Schlafke & Enders, '67; Dvorak, '78). Comparative ultrastructural analysis has also been used to determine the nature of genetic defects affecting preimplantation mouse embryos, such as the $c^{25H}$ mutant (Nadijcka et al., '79).

The anticancer drug cyclophosphamide is a mutagen (Hales, '82), carcinogen (Schmal & Habs, '79), and teratogen (Gibson & Becker, '68; Hales, '82), which can have deleterious effects on reproduction. Using a rat model, it has been shown that chronic low doses of cyclophosphamide

interfere with progeny outcome without obvious effects on the male reproductive system (Trasler et al., '85, '86, '87). A high proportion of the progeny of cyclophosphamide-treated males die in the peri-implantation period, forming implantation sites containing few or no embryonic cells (Kelly et al., '92). In fact, progeny of treated males are affected as early as day 3 of gestation, when a decrease in cell proliferation is apparent (Austin et al., '94).

There are several possible mechanisms by which cyclophosphamide exposure in the male could affect progeny outcome. The most obvious target for an alkylating drug such as cyclophosphamide is the paternal DNA, which is thought to be activated at the 2-cell stage in rats (Telford et al., '90). Cyclophosphamide has been shown to alkylate DNA on the N-7 group of guanine (Benson et al., '88), and may target specific vulnerable sites in the spermatozoal DNA, genetically altering paternal genes coding for proteins required in early embryo cell proliferation. The result of cyclophosphamide exposure is a decrease in embryonic cell number - it is not clear whether this is due to retardation of growth or due to active death of the embryonic cells.

Although little is known about the characteristics of cell death in preimplantation embryos, three modes of developmental cell death in postimplantation embryos have been proposed based on the role of the lysosomes in the dismantling of the cell. The three - apoptosis, autophagy

and non-lysosomal degeneration - are differentiated primarily by their ultrastructural characteristics (Clarke, '90). Although the importance of condensation of nuclear chromatin as a distinguishing feature of apoptosis is currently under debate (Oberhammer et al., '93), apoptosis is characterized by condensation of chromatin at the margin of the nucleus followed by eventual nuclear fragmentation, cytoplasmic condensation and blebbing-off of apoptotic bodies containing intact cell components. In contrast, autophagy is primarily characterized by the formation of numerous autophagic vacuoles, and is sometimes accompanied by mitochondrial and membrane changes. In some cases the nuclei of autophagic dying cells are pyknotic, but this is a minor and rare occurrence compared to the striking nuclear condensation observed in apoptosis. The third type of cell death is non-lysosomal vesiculate degradation that includes non-lysosomal disintegration of the cytoplasm and the "cytoplasmic" type of cell death in which swelling of the organelles precedes the eventual breakdown of the cell (Clarke, '90).

In progeny of male rats exposed to cyclophosphamide in chronic low doses, there is a depletion of embryonic cells in the implantation site on day 7 of gestation (Kelly et al., '92). The cell number in preimplantation blastocysts sired by cyclophosphamide-treated males is only one-half that of controls; the decrease is not selective for inner

cell mass cells (Austin et al., '94). Although there is a decrease in DNA synthesis during preimplantation development (Austin et al., '94), this does not clarify whether all the cells are affected similarly and simply divide slowly or whether there is a heterogeneous effect where some proportion of the embryonic cells die. It is also unclear whether paternal cyclophosphamide exposure affects cell differentiation as well as cell proliferation.

The present study was carried out to search for subcellular evidence of cyclophosphamide toxicity in the embryo to learn more about the mechanisms involved in its eventual death.

## Materials and Methods

### Animals

Adult male (300-315 g) and virgin female (225-250 g) Sprague-Dawley rats were obtained from Charles River Canada Inc. (St. Constant, Québec, Canada) and housed in the McIntyre Animal Centre. Food and water were provided *ad libitum* and animals were maintained on a 14-hr L:10-hr D cycle.

### Treatment and Mating

Male rats were randomly divided into two groups (control and cyclophosphamide-treated). Males were gavaged as previously described (Kelly et al., '92). Treated males received 6.0 mg/kg/day cyclophosphamide (Procytox®, Frank Horner, Montréal, Québec, Canada) and control males received

an equal volume of saline. Males were bred to virgin female rats in proestrus during the fifth week of treatment. Males were not gavaged on the day of their overnight mating, to avoid seminal transmission of cyclophosphamide (Hales et al., '86). The morning after mating (designated day 0 of gestation), females were checked for spermatozoa in the vaginal smear. Sperm-positive females were killed on day 2.5 (8-9 pm), day 3 (10-11 am), and day 4 (10-11 am) of gestation. Embryos were collected from oviducts or uteri using a modified M2 medium (mM2, without phenol red). For all time points the litters from 3 different males were assessed except for day 2.5 control where the litters from 4 males were assessed. For day 2.5, there were 27 embryos in the control group and 21 embryos in the treated group. For day 3.0, there were 17 embryos in the control group and 12 embryos in the treated group. For day 4.0, there were 9 embryos in the control group and 9 embryos in the treated group.

Preparation of Material

Embryos were prepared for transmission electron microscopy using the method of Britton et al. ('90). Embryos were transferred from flushing medium (mM2) to a glass depression slide containing 2.5% glutaraldehyde in 0.1M phosphate buffer (GA) and fixed for 45 minutes at room temperature. The embryos were then washed twice in phosphate buffer, once in 10% bovine serum albumin in

Dulbecco's phosphate buffer (BSAD), and transferred to a dust-free BEEM capsule containing 10 μl BSAD. The embryos were left to settle to the bottom of the BEEM capsule for at least 30 minutes. The capsule was then centrifuged horizontally for 15 minutes at 1800 g at room temperature. Three drops of GA were dropped onto the surface of the BSAD, and the capsule was centrifuged again at 1800 g for 60 minutes. The capsule was then filled with GA and refrigerated overnight at 4°C.

The following morning the BSAD mold containing the embryos was removed from the BEEM capsule after the GA was poured off. The bottom of the capsule was cut away from the rest using a razor blade, and the mold loosened by manually flexing the plastic of the BEEM capsule and using a micropipette with a sealed round end to loosen the mold in the corners.

The mold was transferred to a vial containing phosphate buffer for postfixation with osmium, followed by dehydration and infiltration with Epon. The molds were aligned in the bottom of BEEM capsules and routinely polymerized. Ultrathin sections were cut on a Reichert Ultramicrotome, placed on single-slot grids coated with Formvar, and stained with lead citrate and uranyl acetate. For transmission electron microscopy a Phillips 410 electron microscope was used.

## Results

### *Day 2.5 control*

On the evening of day 2 (day 2.5) 4 litters of embryos sired by 4 different control males were isolated.

Three of the four litters contained embryos with the same morphology. Despite the apparent alignment of apposed surfaces parallel to one another, there was generally very little contact between blastomeres (Fig. 1a,b). The zona pellucida was homogeneous, with irregular inner and outer surfaces. Microvilli were present in moderate numbers and the blastomere surface appeared to contain numerous small irregular indentations, particularly on borders facing other cells (Fig. 1a,b).

The most striking features of these cells were the well-aligned, closely-spaced arrays of up to 30 parallel sheets which were found throughout the cytoplasm, which often had an undulating form in these cells (Fig. 1a-d). These elements were previously identified as cytoskeletal sheets containing a storage form of cytokeratin, the structural protein of intermediate filaments (McGaughey & Capco, '89; Capco et al., '93). The role of these cytokeratin sheets is not presently clear, but it has been postulated that they might hasten trophectoderm differentiation by providing immediately accessible

intermediate filaments, avoiding extensive *de novo* synthesis of cytokeratin protein (Gallicano et al., '92).

The mitochondria were roughly spherical, and appeared to contain marginal cristae, difficult to discern against the matrix (Fig. 1c,d). The matrix density of the mitochondria varied among litters (Fig. 1c,d). Although the differential morphologies of mitochondria may indicate different developmental stages, it should be kept in mind that mitochondrial variation can be due to experimental artifact. There was an even and random distribution of mitochondria throughout the cytoplasm.

Throughout the cytoplasm, and particularly in the marginal regions, clusters of smooth endoplasmic reticulum membranes were found associated with large vesicles, which contained various forms of electron-dense material and may have been autophagic vacuoles (Fig. 1e). The ribosomes appeared as individual dense granules and were abundant throughout the cytoplasm. There was no evidence of typical rough endoplasmic reticulum with associated ribosomes, and Golgi dictyosomes were infrequent.

Nuclei were generally centrally located and elliptical, with a large prominent nucleolus (Fig. 1c,d). The nucleolus was composed of a very dense spherical fibrous body, usually accompanied by surrounding nucleonema. Membrane invaginations were sometimes observed in the nucleoplasm.

One of the 25 embryos from these three litters was

highly fragmented, showing 27 fragments in a single section (Fig. 2a). Each fragment contained condensed cellular components (Fig. 2b). This embryo likely represented a dying oocyte undergoing cytoplasmic fragmentation prior to subsequent degradation.

In one of the four litters, sections contained only two embryos; each displayed only a single blastomere in the sections examined (Fig. 3a). The blastomeres were oval in shape, with microvilli and a few cell membrane loops (Fig. 3a). The background density of the cytoplasm was light. Occasional single cytokeratin sheets were also observed at this stage. Mitochondria were randomly dispersed in the cytoplasm and were darker than the background cytoplasm (Fig. 3b). As in the other two litters, the densities of the matrix and intracristal space were similar so that cristae were difficult to delineate. Areas of clustered tubules represented smooth endoplasmic reticulum (Fig. 3b). Very few ribosome-like granules were seen in the cytoplasm. Small round vesicles containing heterogeneous electron-dense debris were also visible and resembled secondary lysosomes (Fig. 3b). Unfortunately no nuclei were visible in the available sections.

The single blastomere sections are described separately because they cannot be proven to be fertilized, despite the lack of germinal vesicles. Their developmental stage is likely to be considerably earlier than those of the other

three litters. There can be considerable inter-litter variation in the developmental stage of embryos in different females mated on the same night. Previous studies have shown that the average cell number for control embryos collected at this time is 3.5 ± 0.2 cells (Austin et al., '94). Thus the other litters, with sections of embryos containing at least two cells, may be more representative of the average embryo at this developmental time point (Fig. 1a,b).

*Day 2.5 treated*

Embryos sired by treated males were collected on the evening of the second day of gestation.

In the embryos from two of the three litters (Fig. 4a,b), the cytoplasm contains numerous ribosomes (Fig. 4c,d). In these embryos, the cytokeratin arrays were long and undulating (Fig. 4d), and organelle morphology did not differ from control day 2.5 embryos (Fig. 1d).

In one litter, two of the six embryos examined had considerable perivitelline debris and one or more of the cells had clearly lost membrane integrity (Fig. 5a-d). Some membrane was still visible, but discontinuous, with cell contents emptying into the perivitelline space (Fig. 5a,b). Nuclei in various forms of degradation could be observed in the disintegrating regions (Fig. 5a-c). The cells were

round and smooth with varying amounts of microvilli, but often there was a considerable abundance of loops of membrane (Fig. 5d) forming surface blisters extending from the plasma membrane out toward the zona pellucida.

These and the other four embryos from this litter differed from those in the other two litters in cytoplasmic density, number of ribosomes, and condensation of cytokeratin arrays. In these embryos (Fig. 6a), the matrix of the cytoplasm was very light, with very few ribosomes, and the cytokeratin arrays were shorter, sparser, and more multidirectional (Fig. 6b). With the exception of the loss of membrane integrity in two embryos, the morphology of the embryos in this litter (Fig. 6a,b) resembled that of the single-cell control day 2.5 embryos (Fig. 3a,b), rather than that of the other three day 2.5 control litters (Fig. 1a-d).

The other subcellular morphological characteristics of all of the embryos sired by treated males did not differ from one another (Fig. 6a,b; 4a,b) or from the multicellular control day 2.5 embryos (Fig. 1a,b). The variation in cytoplasmic density, ribosome numbers and cytokeratin arrays in one litter of the day 2.5 treated group (Fig. 6b) was also seen in one of the control day 2.5 litters (Fig. 3b). Thus the only notable difference between control and treated groups at this time point was the loss of cell membrane integrity in a minority (2 out of 21; Fig. 5a,b) of the embryos observed.

*Day 3.0 control*

Again considerable interlitter variation was seen in the embryos examined at this developmental stage. Of the three litters examined, sections from one litter contained two 4-cell embryos which had not yet compacted; in the other two litters the embryos were clearly compacted.

The fifteen compacted embryos (Fig. 7a-d) from two of the litters were more likely to represent the average developmental stage at this time point, since previous studies showed that the average cell number in control embryos collected at this time was 9.9 ± 1.0 (Austin et al., '94).

For the first time a blastomere completely enclosed by the others was apparent (Fig. 7b). The blastomeres of these embryos conformed tightly to one another, with multiple contact points along roughly parallel borders, separated by numerous concave indentations of varying width (Fig. 7a,b).

Tight junctions were beginning to form at the outer extremity of intercellular membrane contacts; there was some cytoplasmic darkening just under the surfaces of these contact points which may indicate formation of primary desmosomes (Fig. 7c,d).

Generally organelles were oriented in a column-like formation between the nucleus and the outer surface of the

cell (Fig. 7b). Ribosome granules were numerous and dense, and were scattered individually throughout the cytoplasm among the cytokeratin arrays (Fig. 7e).

The mitochondria were generally round with variable matrix density depending on the litter (Fig. 7c,d). In all cases, cristae crossed as chords around the margins of the mitochondria. Autophagic vacuole-like bodies were found in the column of organelles in the cytoplasm (Fig. 7c,d); these were larger and more numerous than those found in the day 2.5 embryos.

The nuclei were located basally in some cells which appeared to be establishing cytoplasmic polarity (Fig. 7b). Nuclei were still relatively round with multiple nucleoli and nucleonema in almost all cases. Sometimes invaginations of the nuclear membrane were observed.

The two uncompacted 4-cell embryos contained spherical cells with membranes less apposed to one another than those of control day 2.5 embryos (Fig. 8a). The light mitochondria contained marginal cristae and the linear cytokeratin arrays were dense throughout the cytoplasm (Fig. 8b). Although the difference in the density of the mitochondrial matrix and the linearity and number of cytokeratin arrays were different to control day 2.5 embryos, these were the only notable differences; the organelles and their distribution were similar to day 2.5 control embryos (Fig. 1a-d).

*Day 3.0 treated*

Sections from two of the three litters of day 3 embryos sired by treated males only contained a total of 4 embryos (2 each) which were very different to the control day 3 embryos. The sections from the other litter, however, contained 8 embryos which were very similar to control day 3 embryos.

Eight of the embryos evaluated at this time point were sired by one male, and appeared similar to day 3.0 controls (Fig. 9a,b). The number of cells visible in a single section ranged from 2 to 8 cells, fewer, as expected, than in control embryos. There were nascent desmosomes between cells at their outer extremities (Fig. 9a,b). The rare contact points between cells, separated by large gaps, had a noticeable darkening under the membrane which may represent newly forming occluding juxtamembranal homodesmosomes (Fig. 10a,b). Cytokeratin sheets were present in the cytoplasm in scattered parallel arrays of slightly wavy lines (Fig. 10a,b), and like the control day 3.0 embryos, the cytokeratin sheets were not as dense as those seen in day 2.5 embryos. There were numerous individual ribosomes present throughout the cytoplasm, but there was no easily identifiable rough endoplasmic reticulum. With the exception of the cytokeratin sheets and the ribosomes, in many cells the organelles were present in a columnar

orientation between the basally-located nucleus and the apical cell surface (Fig. 9a). This area contained Golgi regions, smooth endoplasmic reticulum, and a large number of relatively small autophagic vacuoles containing electron-dense flocculent material (Fig. 10a,b). The mitochondrial matrices were denser than the cytoplasm, and contained lamelliform septate cristae which were often fused (Fig. 10a,b). As in the control day 3.0 embryos, the basally-oriented nuclei contained round, dense, sometimes-multiple nucleoli, often surrounded with nucleonema structures.

The other four embryos had one, two, four and five cells respectively. These embryos had the light cytoplasm, dark mitochondria and scattered linear cytokeratin arrays (Fig. 11a,c) also observed on day 2.5 in single litters of both control (Fig. 3a,b) and treated (Fig. 6a,b) groups. The one-cell embryo was composed of condensed cell fragments and a single cell which appeared to be blebbing at one end (Fig. 11a). This single large cell contained nuclear fragments and dense round germinal vesicles located just under the plasma membrane (Fig. 11b); this embryo probably represents an unfertilized oocyte undergoing cell degradation.

The low number of embryos in these litters, the fact that this morphology was also observed in both treated and control groups on day 2.5, and the presence in some embryos of cell degradation (by bursting of membranes (Fig. 5a,b) or

fragmentation of cytoplasm (Fig. 11a)) leads to suspicion about whether these embryos represent normal development in any of these groups. The difference observed between litters containing embryos with this morphology and the other litters in their respective treatment groups may reflect sampling bias.

Thus, one litter of day 3.0 embryos sired by treated males was at a developmental stage comparable to control day 3.0 embryos, with the exception that more of the embryos sired by control males had formed inner and outer cells than those sired by treated males. The suspect nature of the other 2 litters assessed at this time point suggests that the first litter is representative of day 3.0 embryos sired by treated males, but this cannot be stated conclusively with the present data.

## Day 4.0 control

The overall shape of the control embryos on the fourth day of gestation was round or oval (Fig. 12a-c). Outer trophectoderm cells were sometimes thin and elongated when adjacent to the blastocoel cavity; the blastocoele was a clear cavity completely enclosed by cells. In all embryos there were inner cells enclosed by the outer trophectoderm cells. There were microvilli and caveolae on the cell membranes facing the zona pellucida. Few microvilli were

found on the blastocoelic surface of the trophectoderm cells.

Some cells were closely apposed with an increased submembrane density along regions of contact, signifying the development of desmosomes (Fig. 13a,b); others had large intercellular spaces of varying width (Figs. 12b; 13b). At the outer extremes of the borders of adjacent cells, there were tight junctions (Fig. 13b,c).

Cytokeratin sheets were present in typical linear arrays in the cytoplasm (Fig. 13a-c). The organization of organelles and appearance of mitochondria in the cytoplasm ranged through three general phenotypes. It should be noted that the phenotypes were litter-specific and may thus represent interlitter variation in developmental stage.

In the first phenotype (Figs. 12a; 13a; 14a), there were widespread, numerous granules interspersed with the cytokeratin sheets. Mitochondria were rounded and mainly clustered in the perinuclear and perimeter regions. As in the day 3 embryos, only sparse cristae crossed the mitochondria as marginal chords. A matrix lighter than background also characterized the mitochondria of these embryos.

In the second phenotype (Fig. 12b; 13b; 14b), the cytoplasm contained areas of cytokeratin sheets, where there were no organelles, and a separation of the ribosomes, which began to cluster around organelles. In this phenotype the

mitochondria were generally more oval than round, with lamelliform septate cristae crossing both as chords and transversely, and occasionally with villous cristae. Some areas of the villous cristae were enlarged and fused with one another, forming bubble-like areas (Fig. 14b). The expanded intracristal areas contrasted with the matrix, which was denser than background.

In the third phenotype (Figs. 12c; 13c, 14c), there was a general separation of areas of cytokeratin sheets with few interspersed ribosomes. The mosaic of multidirectional cytokeratin arrays was striking in contrast to the denser organelle-rich areas, filled with ribosome clusters, which were located around the nucleus and cell margins, particularly apically and basally (Fig. 13c). Mitochondria in this phenotype had a matrix with approximately the same density as the background and had become elongated and thin, with regular transverse lamelliform cristae (Fig. 14c).

Many clusters of membranes forming smooth endoplasmic reticulum were found throughout the cytoplasm in all three phenotypes, along with multiple Golgi complex areas, particularly near the nucleus (Fig. 13a,b,c). Networks of widely-spaced membranes lined with ribosomes, forming the rough endoplasmic reticulum, were often situated adjacent to mitochondria. Numerous small vesicles were found in the cytoplasm, along with round large autophagic vacuoles full of a variety of dark and light vesicles, ribosomes,

membranes, and myelin whorls (Fig. 14a,c).

Nuclei contained membrane invaginations and dense chromatin around the margin. The sometimes multiple nucleoli were smaller than in the day 3 control embryos, with more extensive nucleonema structures (Fig. 12a,b,c). There was no apparent difference between the nuclear structure of the trophectoderm cells and that of the inner cell mass cells.

*Day 4.0 treated*

Embryos sired by cyclophosphamide-treated males which were isolated on the fourth day of gestation could be separated into two main phenotypes. The first comprised 3 embryos lacking a ring of outer cells (Fig. 15a-c), in sharp contrast to the day 4 controls where all embryos had a ring of outer cells. The second phenotype comprised six embryos which did have an enclosing ring of trophectoderm cells (Fig 18a,b). It should be made clear that, unlike the control day 4 embryos where phenotypic differences appeared to be litter-specific, not all the embryos from a given day 4 treated litter necessarily fell into the same phenotypic category. Thus, embryos in the same litter could have different phenotypes.

In the embryos in which a trophectoderm was not formed, the outer shape of the embryo was oval rather than spherical

(Fig. 15a-c). Cell membranes were characterized by indentations and sparse microvilli. Extreme pole regions of occasional cells had more dense patches of microvilli. There was considerable blistering on the surfaces between cells and on some outer surfaces, in contrast to the control embryos at this time point. The component cells had varying cytoplasmic densities, and very few primary desmosomes (Fig. 16b,c; 17a), separating large gaps between neighbouring cell membranes.

In most cells the organelles were clustered around the nucleus and at the periphery (Fig. 16a-c). Cytokeratin sheets were visible in arrays, the number and organization of which varied considerably among cells. In some cells, the cytokeratin sheets were beginning to segregate from the ribosome clusters and organelles. Patches of smooth endoplasmic reticulum were present (Figs. 16a; 17b), with occasional Golgi areas also seen in the cytoplasm. Ribosomes were gathered around organelle groups. Occasional clusters of ribosomes were present among the individual ribosomes scattered around the cytoplasm.

The mitochondria were an irregular circular or cylindrical shape (Fig. 16a-c; 17d). The few cristae were sometimes fused, similar to those in the day 4 control embryos. The matrix was the same density as the background for the majority of mitochondria, but occasionally, in the same cells, darker mitochondria with villous cristae were

seen, again similar to those seen in the advanced day 4 control embryos.

Large numbers of autophagic vacuoles containing a variety of components were present (Fig. 16a-c). Ribosome-like granules were sometimes present in autophagic vacuoles (Fig. 17c); cells with increased cytoplasmic density had extensive regions full of these autophagic vacuoles (Fig. 17c).

Nuclei were located eccentrically in the cells, and often clumps of nuclear material were seen, separate from the larger nucleus (Fig. 16a,c); these may have been tangential sections of "fingers" of chromatin extending from the nucleus. The nuclei contained nucleoli in a wide range of numbers and sizes, and usually a nucleonema structure was present around the nucleolus. Membrane invaginations were sometimes seen in the nucleoplasm.

The majority of the embryos examined sired by treated males had the second phenotype, with a sealed outer trophectoderm ring of cells forming an oval shape (Fig. 18a,b). The cells in these embryos had the same morphology as the earliest phenotype of control day 4 embryos (Figs. 12a; 13a; 14a). Where the blastocoel cavity was more extensive, adjacent trophectoderm cells were elongated and thin. Junctional complexes joined cells at the outer extremes of the borders of adjacent cells (Fig. 14a-c). Desmosomes were present at cell contact points (Fig. 19a-c).

Inner cells were in closer contact with trophectoderm than with one another.

The cytoplasmic density was approximately the same for all the cells; the thin elongated trophectoderm cells were slightly darker than the inner cells. Organelles were again found clustered around the nucleus and the outer membrane. Cytokeratin sheets were arrayed typically (Fig. 19a-c), but were considerably longer in some cells than in previous stages. Ribosomes were numerous and widespread, gathered in clusters around organelles and in the cytoplasm; individual ribosomes were also present in the cytoplasm. Wide rough endoplasmic reticulum networks were less extensive than in control day 4 embryos.

The round or cylindrical mitochondria had a lighter matrix than background, with mainly transverse cristae (Fig. 19a-c). As in the other phenotype, there were numerous very large autophagic vacuoles containing vesicles, membranes, and myelin figures (Fig. 19a-c). One embryo was notable for autophagic vacuoles filled with ribosome-like granules (Fig. 19c). In embryos where the blastocoel cavity was more extensive, the autophagic vacuoles were larger. The autophagic vacuoles were smaller but more plentiful than the isolated extremely large round vacuoles seen in control day 4 embryos.

The nuclei were located eccentrically; the direction was not consistent (Fig. 18a,b). The often multiple

nucleoli were small with an extensive nucleonema.

In one day 4.0 embryo sired by a treated male, an inner cell with two areas of nuclear material and considerable nuclear condensation was budding off condensed bodies (Fig. 20b,d,f); this was likely a cell undergoing apoptosis. Similarly, in one control day 4.0 embryo there was a cell almost entirely isolated from the others in the blastocoele cavity (Fig. 20a). This cell contained many autophagic vesicles; nuclear fragments containing condensed chromatin could be observed within the autophagic vesicles (Fig. 20c,e). The extensive formation of blebs of condensed cytoplasmic material and autophagic vesicles strongly suggested that this cell was undergoing apoptosis.

Thus, the majority of embryos sired by treated males appeared to differentiate properly, forming inner and outer cell lineages and a blastocoele cavity. Inner cell mass cells were apparent in the day 4 embryos sired by treated males. This confirms previous findings that inner cells were present in the blastocyst (Austin et al., '94), and that the selective loss of inner cell mass-derived cells in the implantation site on day 7 was not due to a selective preimplantation loss of inner cells. Clearly there were embryos sired by cyclophosphamide-treated males which could perform the complex cellular events required for blastocoele formation.

## Discussion

Although there were a few exceptional litters in which embryos sired by treated males manifested developmental difficulties from the earliest stages and did not appear to develop between the day 2.5 and day 3 time points, it would appear that the general decrease in cell proliferation occurred without a concomitant slowing of the morphological changes that occur during preimplantation development. By day 4, however, some embryos sired by treated males appeared not to have proliferated sufficiently to form inner and outer cells. These embryos appeared to fail to respond to normal differentiative cues parallel to the control embryos, and may have had insufficient cells for further development.

There is no gross morphological defect that could clearly be singled out as unique to the embryos sired by cyclophosphamide-treated males. The present experiment has shown that the majority of preimplantation embryos sired by cyclophosphamide-treated males were capable of cell differentiation and blastocoele formation despite the drastic reduction in cell number demonstrated in previous studies (Austin et al., '94). The reduction in the number of embryonic cells may be due to a retardation in cell proliferation between day 2.5 and day 3.0 of gestation.

Embryonic death may be more rapid in those embryos which fail to form inner and outer cell populations.

The embryonic stages observed in embryos sired by control males in this experiment correlate with previous findings in the literature for rat preimplantation development (Sotelo & Porter, '59; Schlafke & Enders, '67; Dvorak, '78). The columnar organization of cytoplasmic organelles observed in compacted day 3.0 control embryos was previously described in 8-cell rat embryos (Izquierdo & Vial, '62; Schlafke & Enders, '67; Enders, '71; Lois and Izquierdo, '84). The unique nucleolar structure and timing of appearance of nucleonema in the nucleus of the blastomere were the same as that previously observed (Szollosi, '66; Schuchner, '70). The present observation of multiple stages of development with progressive segregation of cytokeratin sheets from other clustered cytoplasmic components in the day 4 embryos sired by control males correlates with similar observations in rat embryos by other investigators (Schlafke & Enders, '67, '73; Enders, '89). Similarly, the transformation of the mitochondria from the round phenotype typical of the unfertilized oocyte, containing marginal septate lamelliform cristae, to the elongated form found in blastocysts, containing a mixture of lamelliform and villiform cristae, has also been previously described in the literature (Enders, '71; Schlafke & Enders. '73; Dvorak, '78; Enders, '89).

A certain amount of normal apoptotic physiological cell death occurs in blastocysts, and has been proposed to be mediated by hydrogen peroxide in the blastocoelic fluid (Pierce et al., '91). Large autophagic vacuoles similar to those seen in both control embryos and those sired by treated males in the present experiment were stained for acid phosphatase activity in control embryos in a previous study (Schlafke & Enders, '73), and thus were identified as secondary lysosomes. These vacuoles are present in normal embryos of both rat and mouse and are presumed to digest not only the changing products of the differentiating cell but the debris of the programmed apoptotic death of other blastomeres (El-Shershaby & Hinchcliffe, '74; Pierce et al., '90). The number of these vacuoles seemed increased, however, in the some cells of embryos sired by cyclophosphamide-treated males which did not form inside and outside cells by day 4. Clearly, this autophagic cell degradation occurring in the day 4 embryos was different from the observed sporadic apoptotic death of cells isolated in the blastocoelic cavity.

The early acting recessive lethal mutation lethal yellow ($A^{y}/A^{y}$) in the mouse bears considerable resemblance to the present model. In ($A^{y}/A^{y}$) homozygous mouse embryos the cells undergo retardation and arrest during cleavage in the preimplantation embryos. The manifestations of the defects depend on the genetic background, but apparently defects are

first noted in the trophectoderm cells (Papaioannou & Gardner, '92). Both tissues, however, are affected eventually and the embryos die at the late blastocyst stage (Pedersen, '74). Homozygous blastocysts fail to hatch and form blebs and fragments (Pedersen, '74), but can elicit a uterine decidual response (Eaton & Green, '62, '63). Even if the embryos are allowed to continue development in the reproductive tract of prepubertal females without implantation cues, they fail to thrive, indicating that the lethality is not stage-specific for implantation (Papaioannou & Gardner, '92). The authors have suggested that this may reflect the exhaustion of a maternal general-function gene product synthesized before fertilization, without its concomitant synthesis from the embryonic genome (Papaioannou & Gardner, '92). The $A^{y}$ mutation disrupts an RNA-binding gene, *Raly*, which is normally expressed at the two-cell stage; the lack of *Raly* protein has been proposed as the cause of embryo death (Michaud et al., '93). At both the ultrastructural and the light microscope level the exclusion of blastomeres from the ($A^{y}/A^{y}$) embryos at the morula and blastocyst stage has been observed (Pedersen, '74; Calarco & Pedersen, '76). The exclusion of blastomeres is not observed in the rat embryos sired by cyclophosphamide-treated males, but the timing of effects may indicate a similar general function gene as a target for paternal cyclophosphamide damage.

Thus it would appear that there are possibly two selection points, in the preimplantation development of the rat, at which cells of embryos sired by cyclophosphamide-treated males falter. One of these would appear to be just prior to day 2.5 of gestation, when the embryonic genome is first activated (Telford et al., '90), and may be indicative of genetic damage sustained in the embryonic genome after paternal drug exposure. The damage may not be so serious as to affect the differentiation of the embryo, but cause a considerable decrease in cell proliferation.

The other selection point would have to occur sometime after day 4 of gestation since only a minority of the embryos sired by cyclophosphamide-treated males had any signs of death at the day 4 time point. The extensive transcriptional demands of gastrulation in the implanting blastocyst could force the manifestation of further genetic damage from exposure of the paternal genome to cyclophosphamide. Alternatively, it has been proposed that a minimum number of inner cells is required for postimplantation development (Ansell & Snow, '75; Snow et al., '76). The drastic decrease in the number of cells in both lineages in embryos sired by cyclophosphamide-treated males may be prohibitory to post-implantation development.

Acknowledgements

The authors would like to acknowledge the excellent technical assistance of Ms. Marie Ballak, also the helpful suggestions of Dr. A. Ribeiro-da-Silva, Dr. L. Hermo, and Dr. J. Brawer. This project was supported by the Medical Research Council of Canada.

## References

Ansell JD, Snow MHL. The development of trophoblast *in vitro* from blastocysts containing varying amounts of inner cell mass. J Embryol Exp Morphol 1975; 33:177-185.

Benson AJ, Martin CN, Garner RC. *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl]amine, the putative major DNA adduct of cyclophosphamide *in vitro* and *in vivo* in the rat. Biochem Pharmacol 1988; 37:2979-85.

Britton AP, Moon YS, Yuen BH. A simple handling technique for mammalian oocytes and embryos during preparation for transmission electron microscopy. J Microsc 1990; 161:497-499.

Calarco PG, Pedersen RA. Ultrastructural observations of lethal yellow ($A^y/A^y$) mouse embryos. J Embryol Exp Morph 1976; 35:73-80.

Capco DG, Gallicano GI, McGaughey RW, Downing KH, Larabell CA. Cytoskeletal sheets of mammalian eggs and embryos: a lattice-like network of intermediate filaments. Cell

Motil Cytoskel 1993; 24:85-99.

Clarke PGH. Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 1990; 181:195-213.

Dvorak M. The differentiation of rat ova during cleavage. Adv Anat Embryol Cell Biol 1978; 55:1-131.

Dvorak M, Travnik P, Stankova J. A quantitative analysis of the incidence of certain cytoplasmic structures in the ovum of the rat during cleavage. Cell Tiss Res 1977; 179:429-437.

Eaton GJ, Green MM. Implantation and lethality of the yellow mouse. Genetica 1962; 34:106-112.

Eaton GJ, Green MM. Giant cell differentiation and lethality of homozygous yellow mouse embryos. Genetica 1963; 34:155-161.

El-Shershaby AM, Hinchcliffe JR. Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate. J Embryol Exp Morphol 1974; 31:643-654.

Enders AC. The fine structure of the blastocyst. In: The Bi ology of the Blastocyst. Blandau RJ, ed.; Univ. of Chicago Press: Chicago, 1971; 71-94.

Enders AC. Morphological manifestations of maturation of the blastocyst. In: Development of Preimplantation Embryos and Their Environment. Yoshinaga K, Mori T, (eds.); Alan R. Liss, New York: 1989; 211-223.

Gallicano GI, McGaughey RW, Capco DG. Cytoskeletal sheets appear as universal components of mammalian eggs. J Exp Zool 1992; 263:194-203.

Gibson JF, Becker BA. Effect of phenobarbital and SKF 525-A on the teratogenicity of cyclophosphamide in mice. Teratol 1968; 1:393-398.

Hales BF. Comparison of the mutagenicity and teratogenicity of cyclophosphamide and its active metabolites, 4-hydroperoxycyclophosphamide, phosphoramide mustard, and acrolein. Cancer Res 1982; 42:3016-3021.

Hales BF, Smith S, Robaire B. Cyclophosphamide in the seminal fluid of treated males: transmission to females by mating and effect on pregnancy outcome. Toxicol Appl Pharmacol 1986; 84:423-430.

Izquierdo L, Vial JD. Electron microscope observations on the early development of the rat. Zeit für Zellforsch 1962; 56:157-179.

Kelly SM, Robaire B, Hales BF. Paternal cyclophosphamide treatment causes postimplantation loss via inner cell mass-specific cell death. Teratol 1992; 45:313-318.

Kelly SM, Robaire B, Hales BF. Paternal cyclophosphamide exposure causes decreased cell proliferation in cleavage-stage embryos. Biol Reprod (in press).

Lois P, Izquierdo L. Cell membrane regionalization and cytoplasm polarization in the rat early embryo. Roux's Arch Dev Biol 1984; 193:205-210.

McGaughey RW, Capco DG. Specialized cytoskeletal elements in mammalian eggs: structural and biochemical evidence for their composition. Cell Motil Cytoskel 1989; 13:104-111.

Michaud EJ, Bultman SJ, Stubbs LJ, Woychik RP. The embryonic lethality of homozygous lethal yellow mice ($A^Y/A^Y$) is associated with the disruption of a novel RNA-binding protein. Genes Dev. 1993; 7:1203-1213.

Nadijcka MD, Hillman N, Gluecksohn-Waelsch S. Ultrastructural studies of lethal $c^{25H}/c^{25H}$ mouse embryos. J Embryol Exp Morphol 1979; 52:1-11.

Oberhammer F, Fritsch G, Schmied M, Pavelka M, Printz D, Purchio T, Lassmann H, Schulte-Hermann R. Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J Cell Sci 1993; 104:317-326.

Papaioannou VE, Gardner RL. Effects of diapause on lethal *Yellow* ($A^Y/A^Y$) mouse embryos. J Exp Zool 1992; 263:309-315.

Pedersen RA. Development of lethal yellow ($A^Y/A^Y$) mouse embryos in vitro. J Exp Zool 1974; 188:307-320.

Pierce GB, Gramzinski RA, Parchment RE. Amine oxidases, programmed cell death, and tissue renewal. Phil Trans R Soc Lond B 1990; 327:67-74.

Pierce GB, Parchment R, Lewellyn AL. Hydrogen peroxide as a mediator of programmed cell death in the blastocyst.

Differentiation 1991; 46:181-186.

Rossant J, Vijh M, Siracusa LD, Chapman VM. Identification of embryonic cell lineages in histological sections of M. musculus <--> M. caroli chimaeras. J Embryol Exp Morph 1983; 73:179-191.

Schlafke S, Enders AC. Cytological changes during cleavage and blastocyst formation in the rat. J Anat 1967; 102:13-32.

Schlafke S, Enders AC. Protein Uptake by Rat Preimplantation Stages. Anat Rec 1973; 175:539-560.

Schmahl D, Habs M. Carcinogenic action of low-dose cyclophosphamide given orally to Sprague-Dawley rats in a lifetime experiment. Int J Cancer 1979; 23:706-712.

Schuchner EB. Ultrastructural changes of the nucleoli during early development of fertilized rat eggs. Biol Reprod 1970; 3:265-274.

Snow MHL, Aitken J, Ansell JD. Role of the inner cell mass in controlling implantation in the mouse. J Reprod Fert 1976; 48:403-404.

Sotelo JR, Porter KR. An electron microscope study of the rat ovum. J Biophys Biochem Cytol 1959; 5:327-342.

Szollosi D. Nucleolar transformation and ribosome development during embryogenesis of the rat. J Cell Biol 1966; 31:115A.

Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a

comparison of several species. Mol Reprod Dev 1990; 26:90-100.

Trasler JM, Hales BF, Robaire B. Paternal cyclophosphamide treatment of rats causes fetal loss and malformations without affecting male fertility. Nature 1985; 316:144-146.

Trasler JM, Hales BF, Robaire B. Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. Biol Reprod 1986; 34:275-283.

Trasler JM, Hales BF, Robaire B. A time-course study of chronic paternal cyclophosphamide treatment in rats: effects on pregnancy outcome and the male reproductive and hematologic systems. Biol Reprod 1987; 37:317-326.

Figure 1

Electron micrographs of day 2.5 embryos sired by control males. Representative embryos sired by two different males are shown in (a) and (b). Cells in the same embryos (c,d) contained nuclei (N) with dark round nucleoli, cytokeratin arrays (C), and mitochondria (m). Vesicles filled with flocculent material were associated with smooth endoplasmic reticulum (sER), shown in (e). Magnification (a,b): 900x; (c,d,e): 6100x.

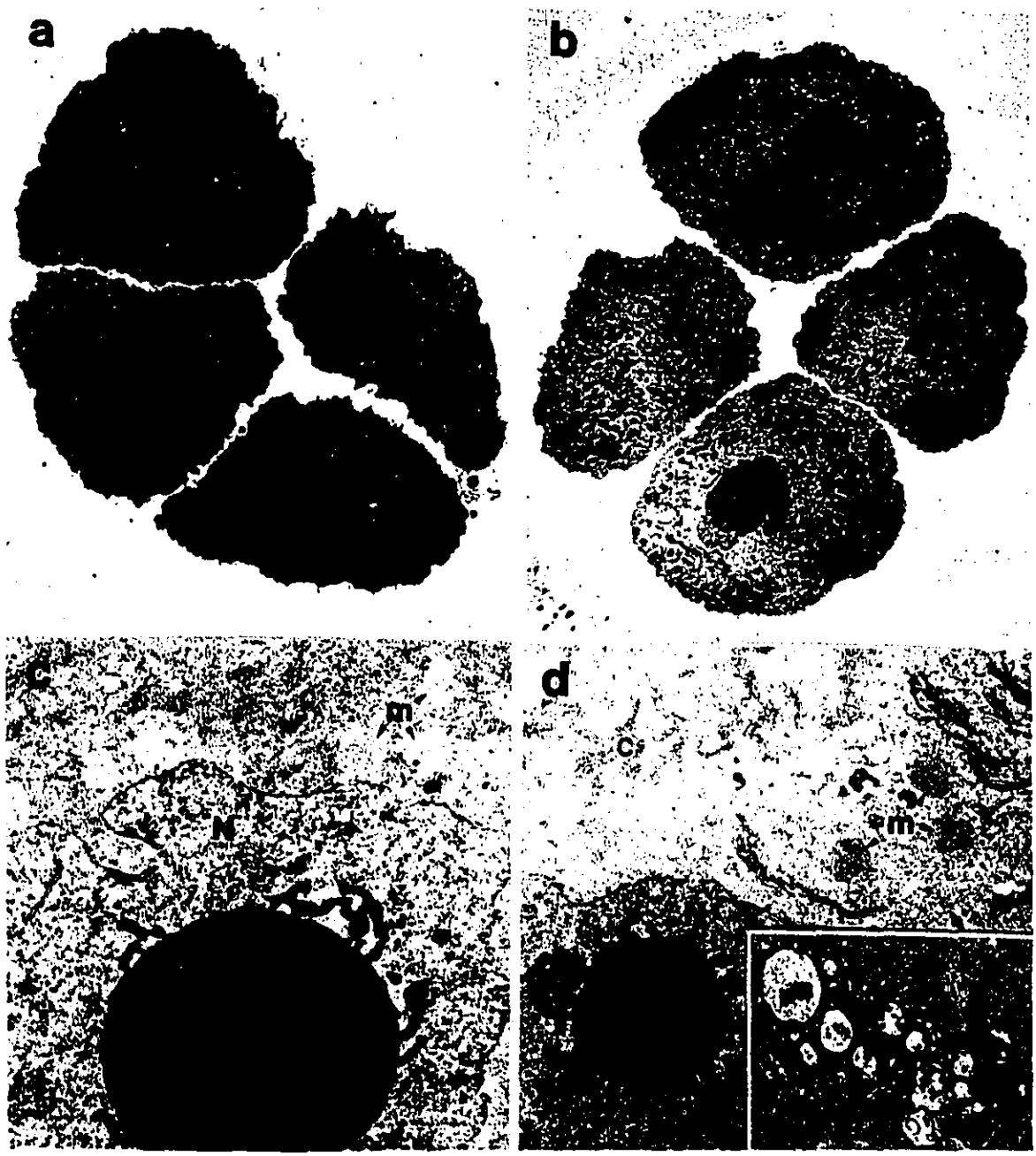

a
b
c
m
N
d
c
m

Figure 2

Electron micrographs of a fragmented day 2.5 embryo sired by a control male. Twenty-seven fragments were present in a single section (a). One fragment (asterisk) is shown at higher magnification in (b). Cytokeratin arrays (C) and one mitochondrion (m) were seen against a dense cytoplasmic background. Magnification (a): 900x; (b): 10200x.

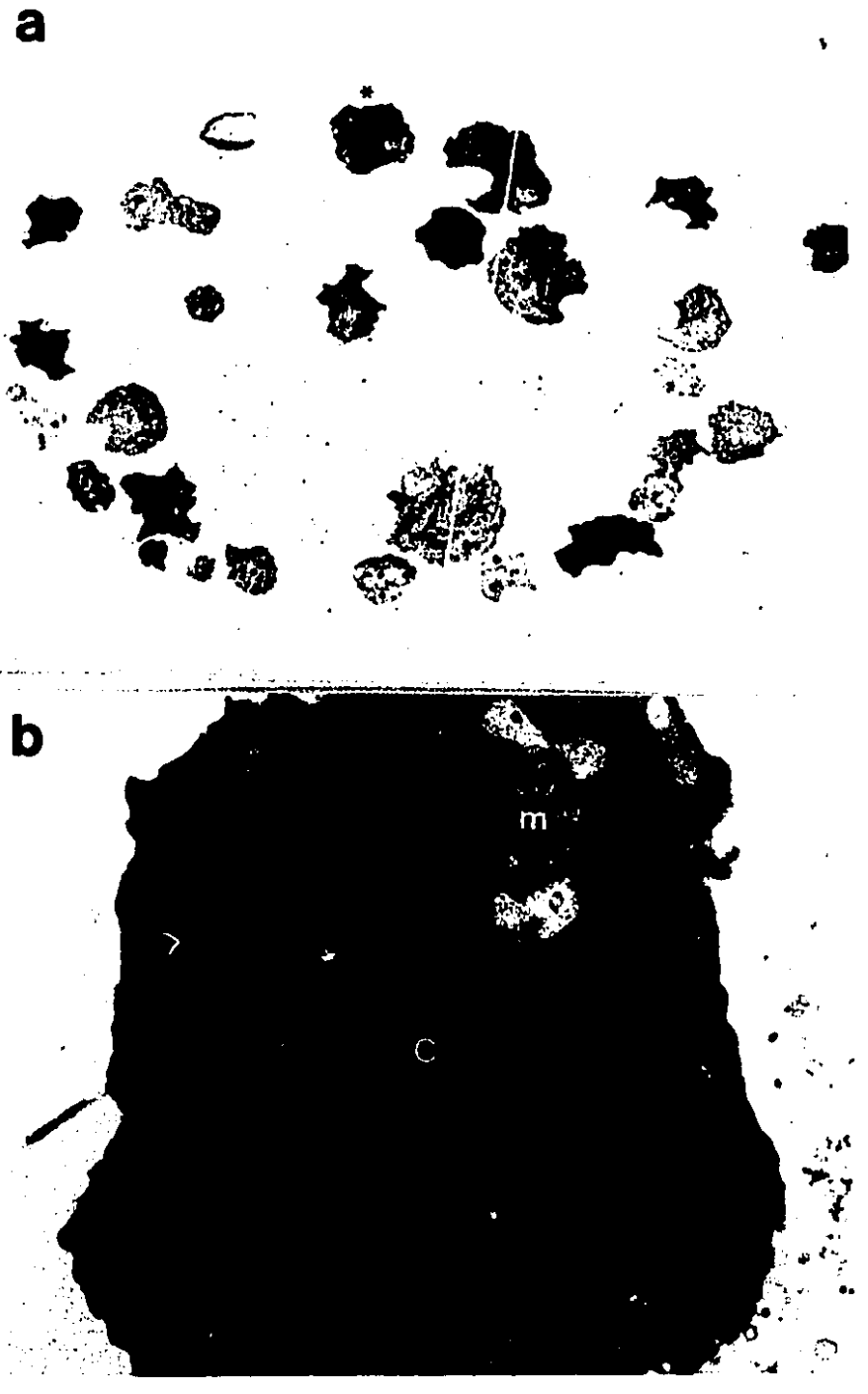

a
b
m
C

Figure 3

Electron micrographs of a one-cell day 2.5 embryo sired by a control male. Numerous membrane loops (arrowheads) were seen at the periphery of the single cell (a). Note the random distribution of mitochondria (m), smooth endoplasmic reticulum (sER), autophagic vacuoles (AV), and cytokeratin arrays (C) against the light cytoplasmic background (b). Magnification (a): 2120x; (b): 4400x.

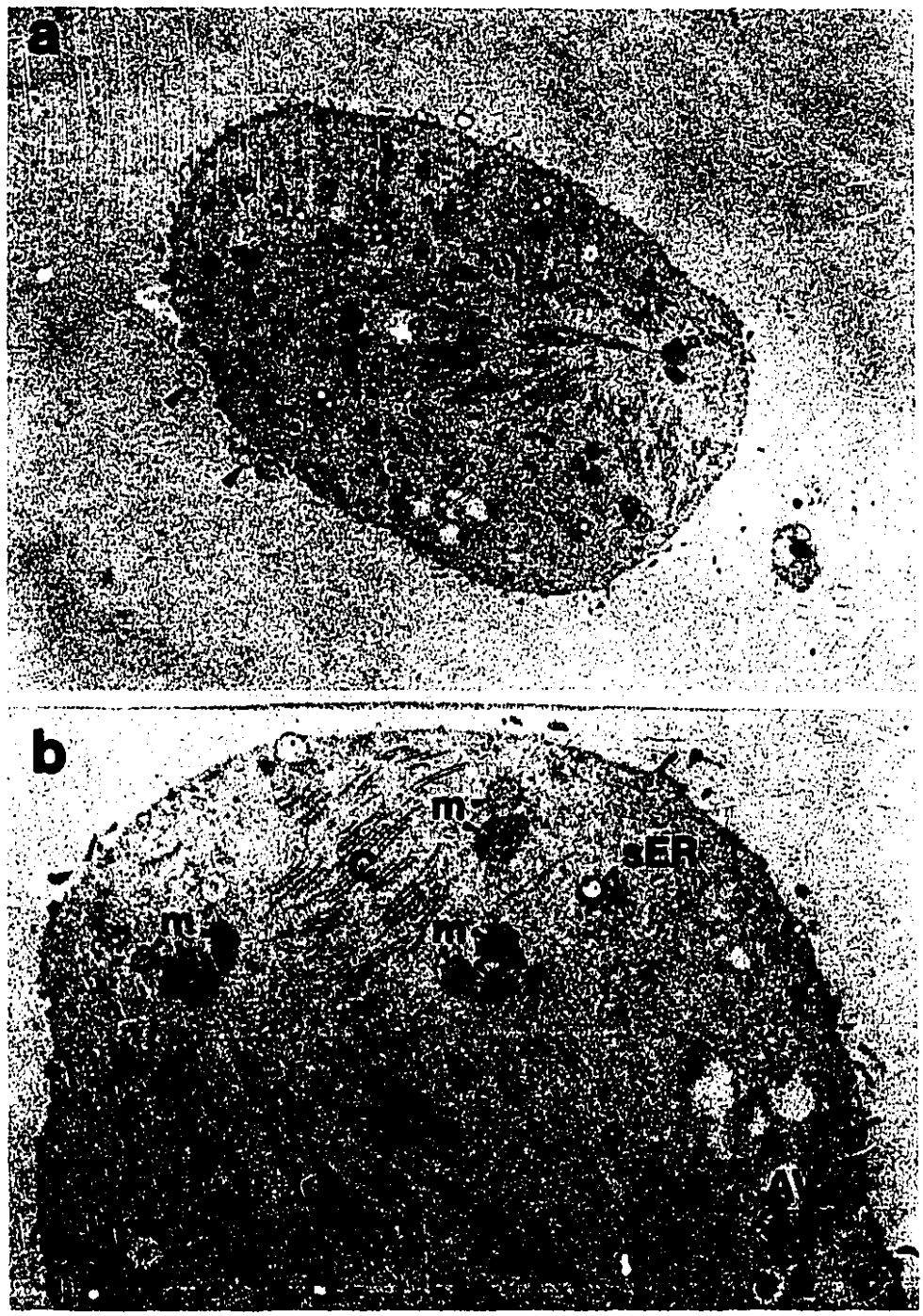

a
b
m
sER
n
m

Figure 4

Electron micrographs of representative day 2.5 embryos sired by two different cyclophosphamide-treated males (a,b). Cells in the same embryos (c,d) contained nuclei (N) with dark round nucleoli, cytokeratin arrays (C), mitochondria (m), and smooth endoplasmic reticulum (sER). Magnification (a,b): 900x; (c,d): 6100x.

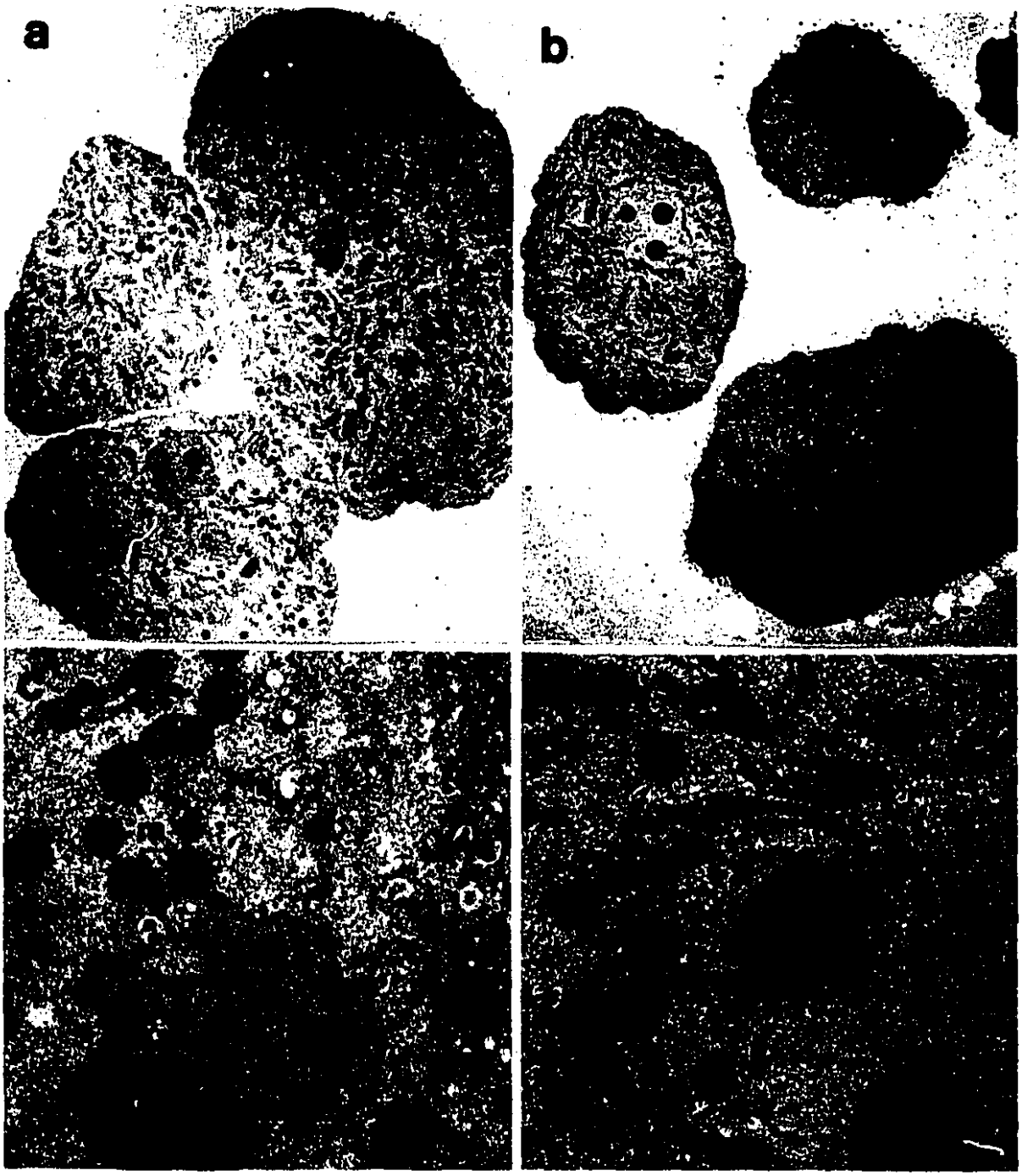

a
b

Figure 5

Electron micrographs demonstrating loss of membrane integrity in the two day 2.5 embryos sired by a cyclophosphamide-treated male (a,b). Cell contents spilled out into the peri-vitelline space (filled curved arrows). Fragments of membrane were still present (arrowheads), and disintegrating nuclear material was observed (open arrows) in the broken cells. In one embryo (b), a metaphase chromosome (Ch) was visible in an adjacent blastomere. Higher magnification of the broken cells (c,d) showed a large nuclear envelope (large filled arrow) around nuclear material (N). The broken cell areas contained swollen mitochondria (m) and scattered individual cytokeratin sheets (small arrows) in the cytoplasm which contrasted with the stacked cytokeratin arrays (C) in adjacent cells. Membrane loops (L) were present on the facing surfaces of adjacent cells (d). Magnification (a,b): 900x; (c,d): 6100x.

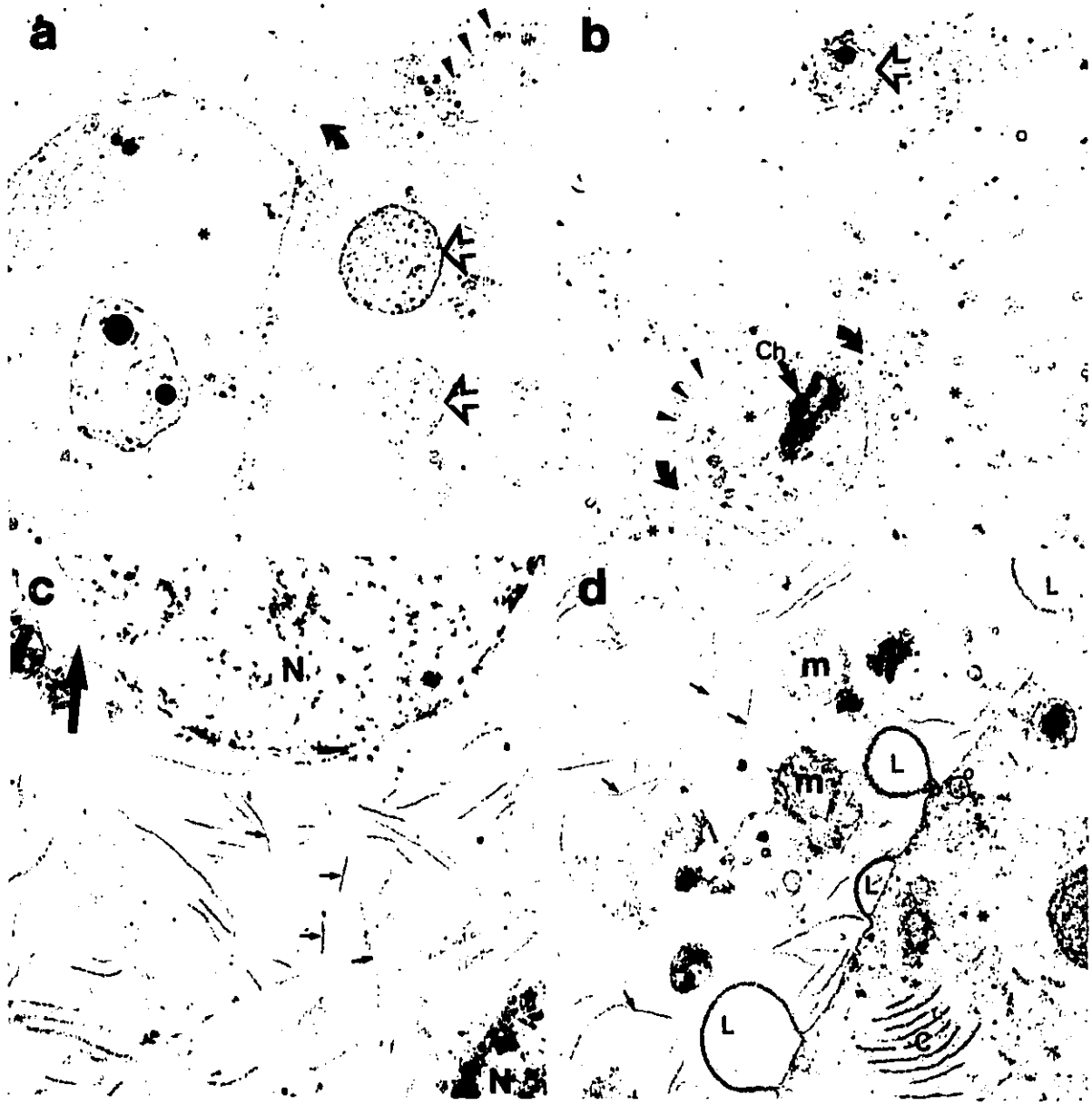

a
b
Ch
c
N
N
d
L
m
m
L
L
L
c

Figure 6

Electron micrographs of a representative day 2.5 embryo (a), a littermate of those in Fig. 5. At higher magnification (b), the cytokeratin arrays (C), smooth endoplasmic reticulum (sER), and dense mitochondria (m) were visible against the light cytoplasmic background. Magnification (a): 900x; (b): 10200x.

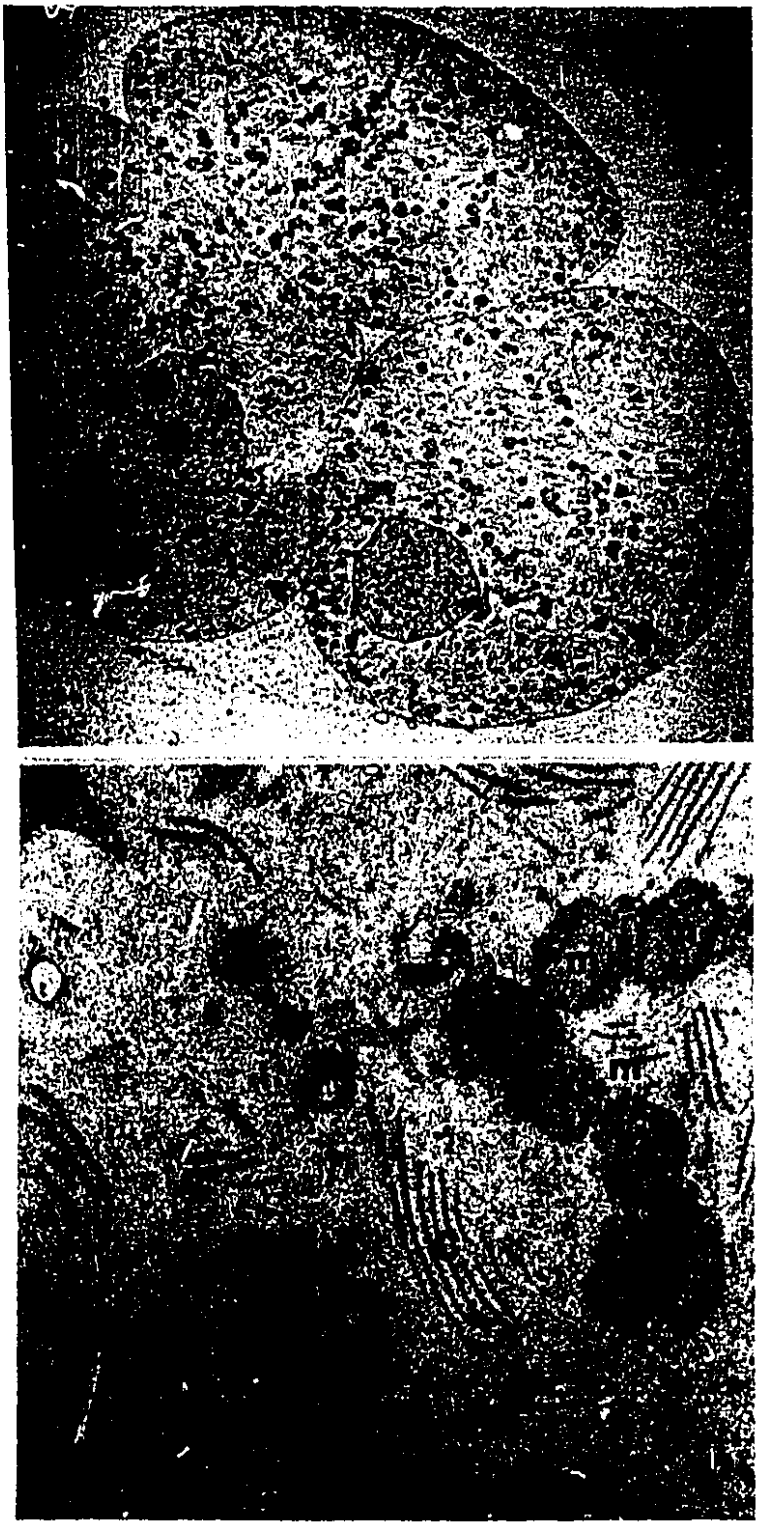


Figure 7

Electron micrographs of representative day 3.0 embryos sired by two different control males (a,b). There is a region completely enclosed by cells contained an inner cell (**) in one embryo (b); the other (a) contained cell debris (*). Organelles were clustered around the nucleus (a) or formed a column between the nucleus and the outer surface (b). Higher magnification of cells in the same embryos (c,d) demonstrated autophagic vesicles (filled arrows), nuclei (N), mitochondria (m), and smooth endoplasmic reticulum (sER). In (e), dense ribosomes (small filled arrows) were interspersed among the sheets of the cytokeratin arrays (C). Magnification (a,b): 900x; (c,d): 6100x; (e): 17700x.

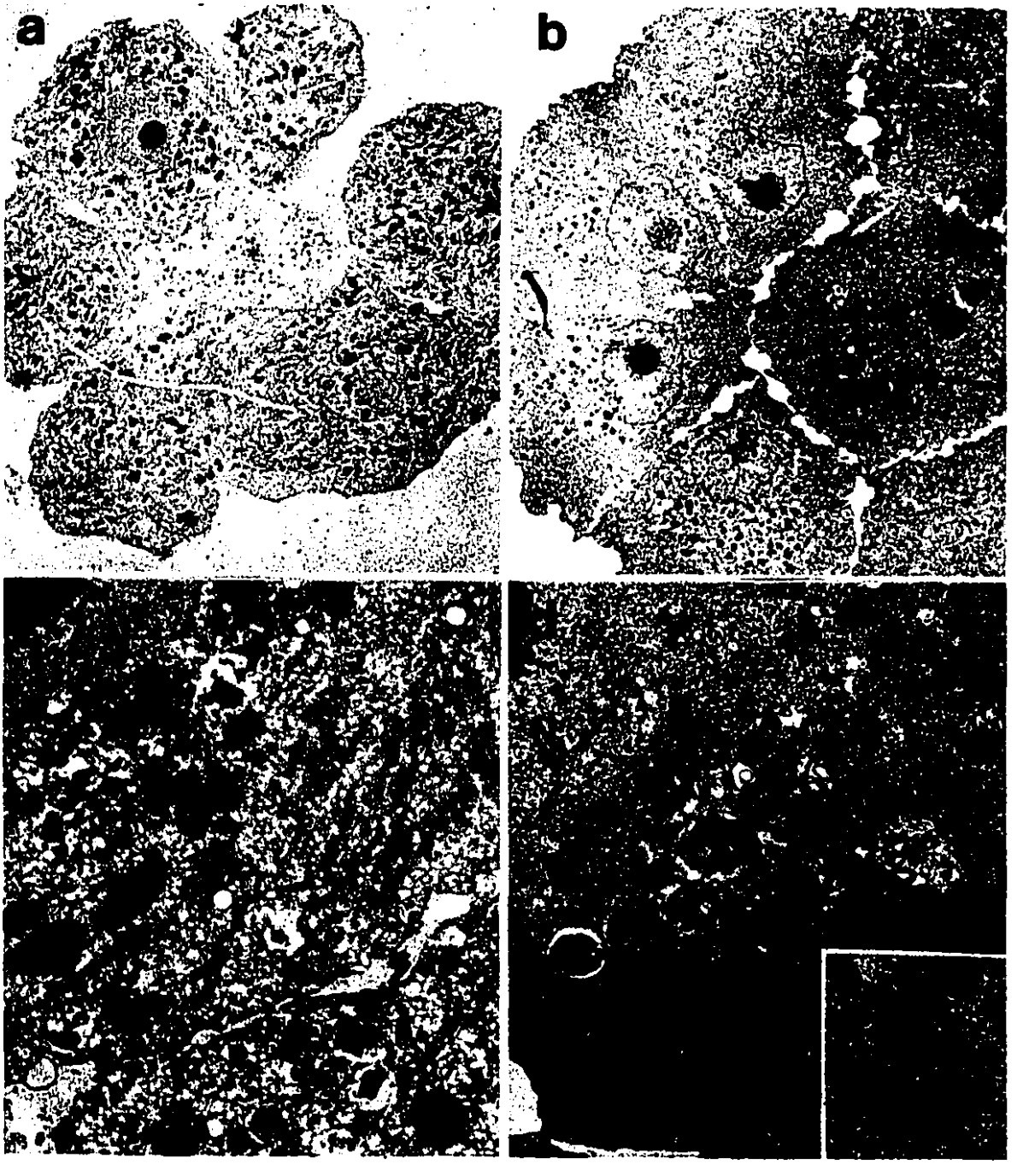

a
b

Figure 8

Electron micrographs of a representative uncompacted day 3.0 embryo sired by a control male. Cells were separated for the most part by gaps (arrowheads); only minimal regions of contact (arrows) were present (a). Within the cell (b), the mitochondria (m) had light matrices, the cytokeratin sheets (C) were dense and linear, and smooth endoplasmic reticulum (sER) was present. The cells shown (b) contained a dense cytoplasmic region (thick filled arrow) not bound by a membrane. Magnification (a): 471x; (b): 1350x.

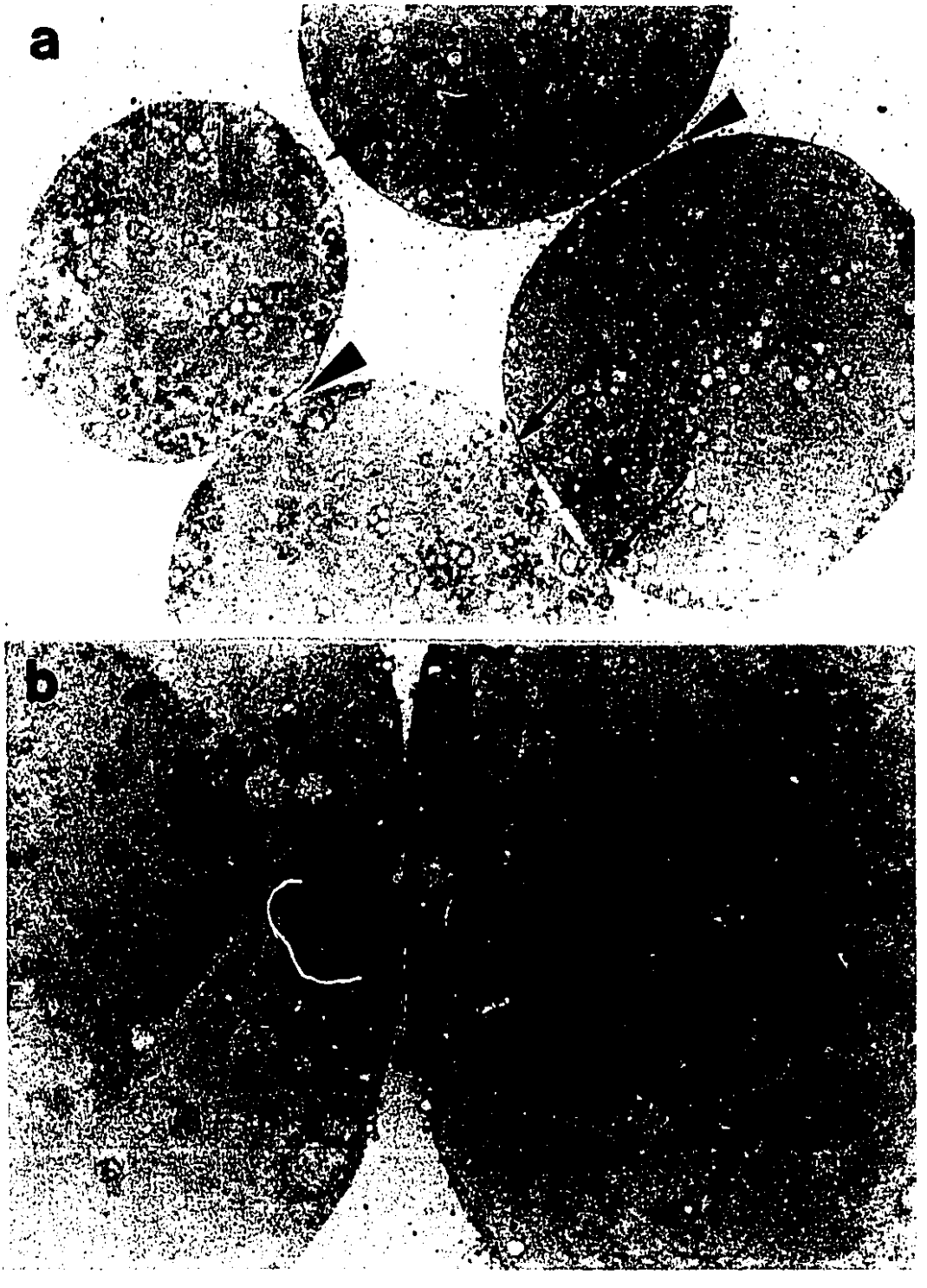

a
b

Figure 9

Low magnification electron micrographs of representative day 3.0 embryos sired by a cyclophosphamide-treated male (a,b). The compacted embryos had cell junctions at the outer extremities of the cells (arrowheads), even when only four cells were present (b). Magnification 900x.

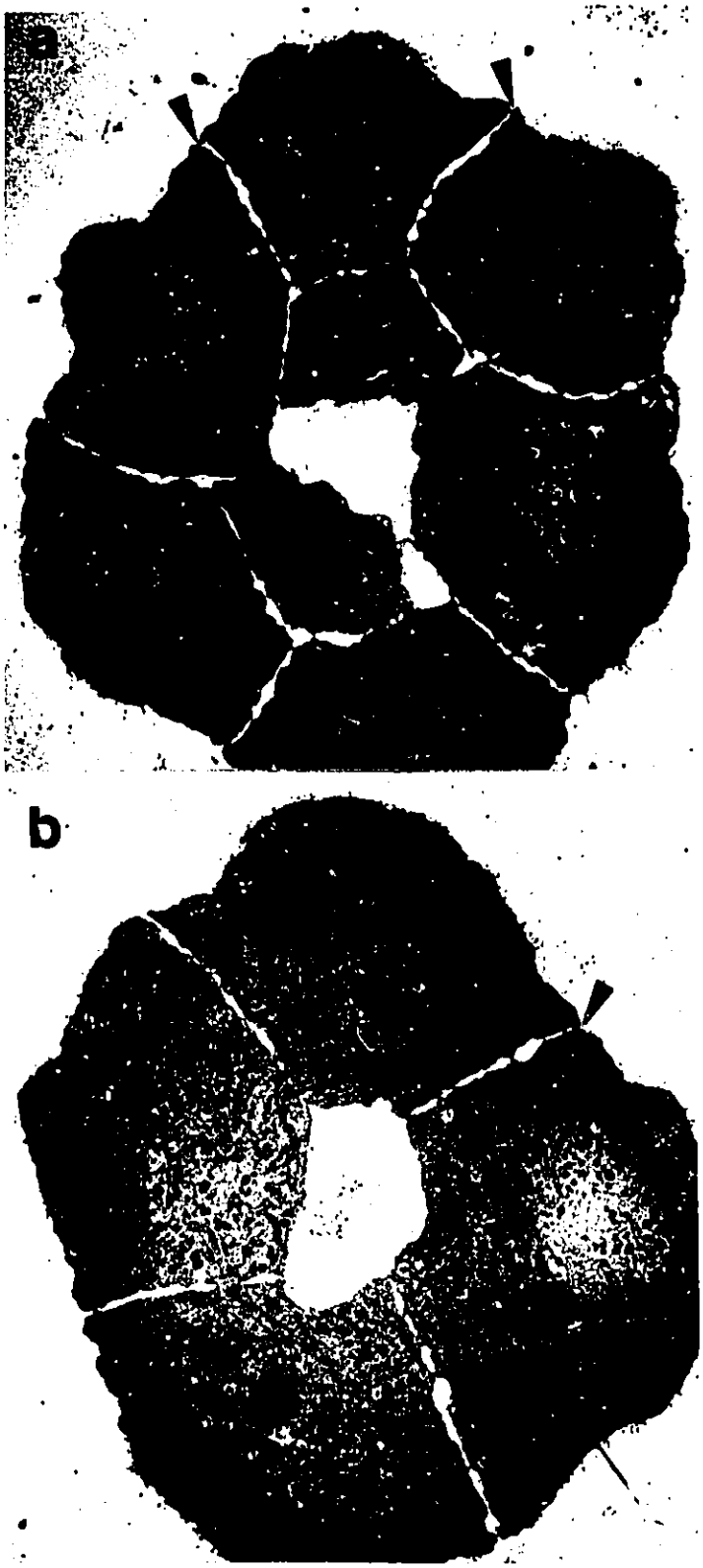

a
b

Figure 10

Electron micrographs demonstrating cytoplasmic organization of day 3.0 embryos sired by a cyclophosphamide-treated male (a,b). Nascent desmosomes (arrowheads) were visible at cell contact points. Note the dark mitochondria (m), autophagic vesicles (filled arrows), and slightly undulating cytokeratin arrays (C). Nuclei (N) were usually not centrally located. Magnification 6100x.

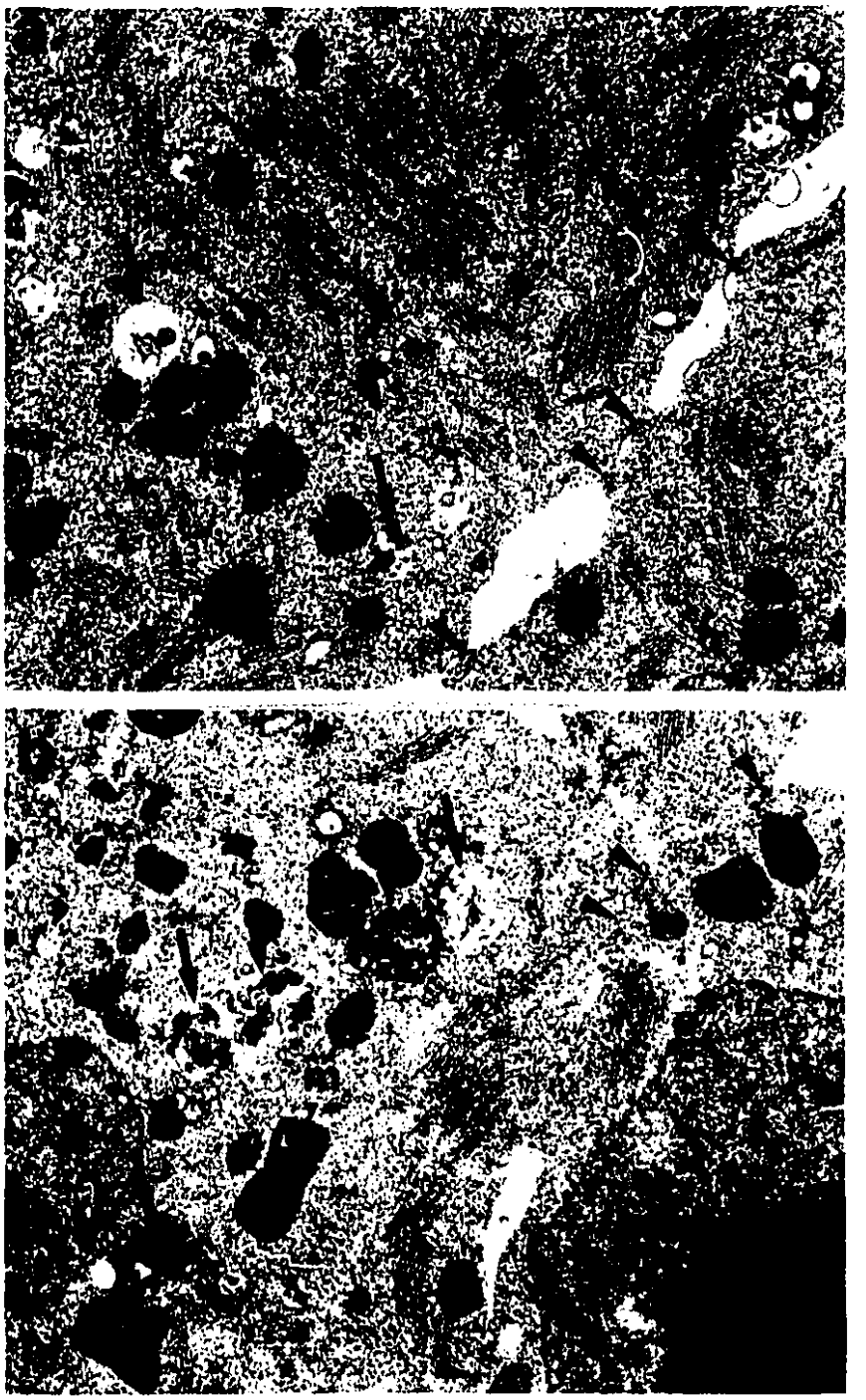


Figure 11

Electron micrographs demonstrating alternate morphology of one-cell and four-cell day 3.0 embryos sired by cyclophosphamide-treated males. The one-cell embryo appeared to be budding off at one end (curved filled arrow) and cell fragments (arrowheads) were visible within the zona pellucida (Z). Higher magnification of the embryo in (a) revealed germinal vesicles (filled straight arrows) under the cell membrane. The cell fragments contained cytoplasmic elements including mitochondria (m), autophagic vacuoles (AV) and enlarged vesicles (arrowheads). The four-cell embryo (c) demonstrated aligned cell membranes. At higher magnification (d), a nascent desmosome (arrowhead) was present at cell contact points. Extensive clusters of smooth endoplasmic reticulum (sER) were present in the cytoplasm, along with dark mitochondria (m), small autophagic vacuoles (AV) and sparse cytokeratin arrays (C). Magnification (a,c): 900x; (b,d): 10200x.

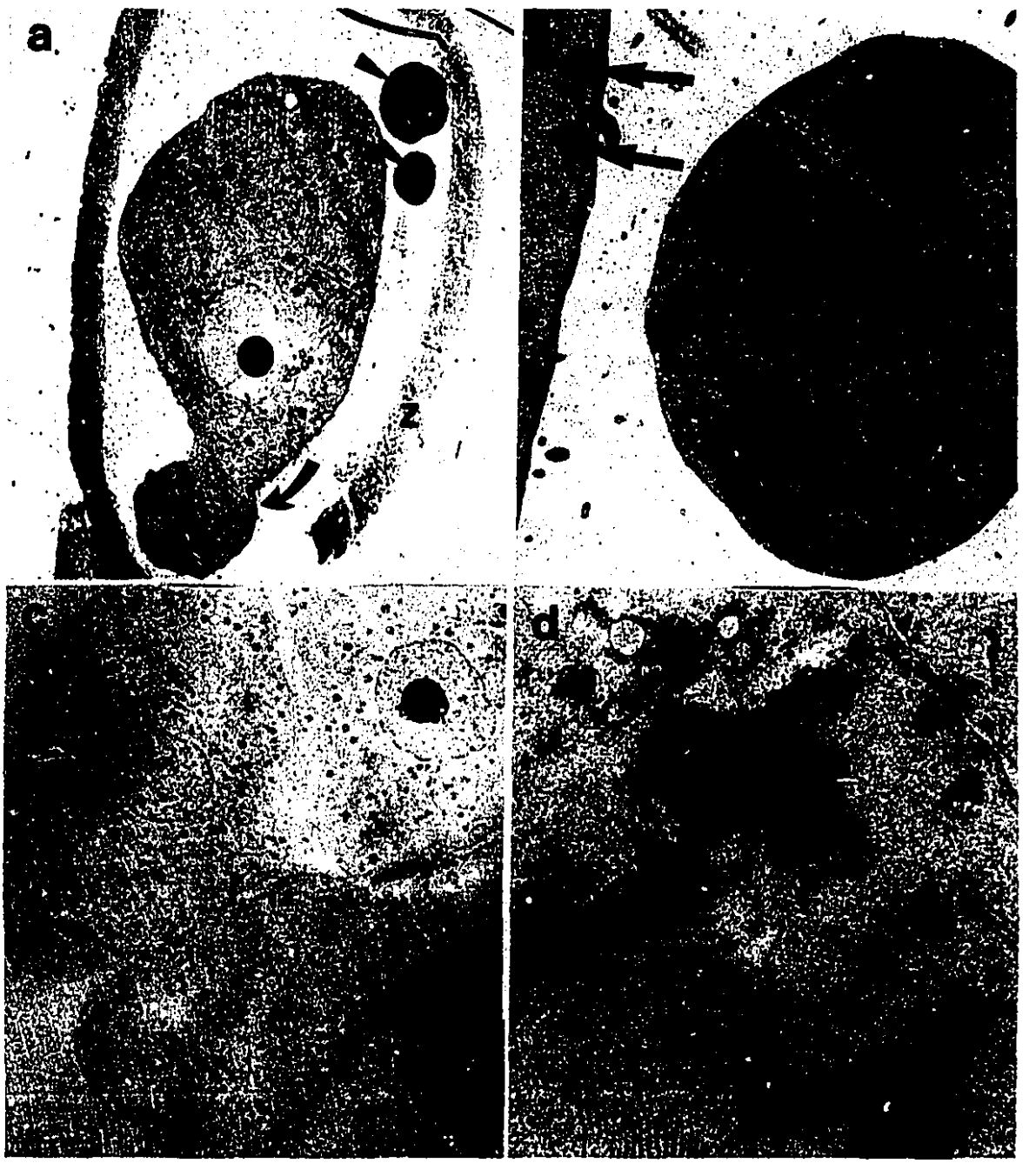

a
d

Figure 12

Low magnification electron micrographs demonstrating the three phenotypes of day 4.0 embryos sired by control males (a,b,c). In all three phenotypes, the surrounding sealed ring of trophectoderm cells enclosed the mass of inner cells (ICM). The blastocoele cavity (Bc) is visible in the plane of the sections in (a) and (c). Magnification 900x.

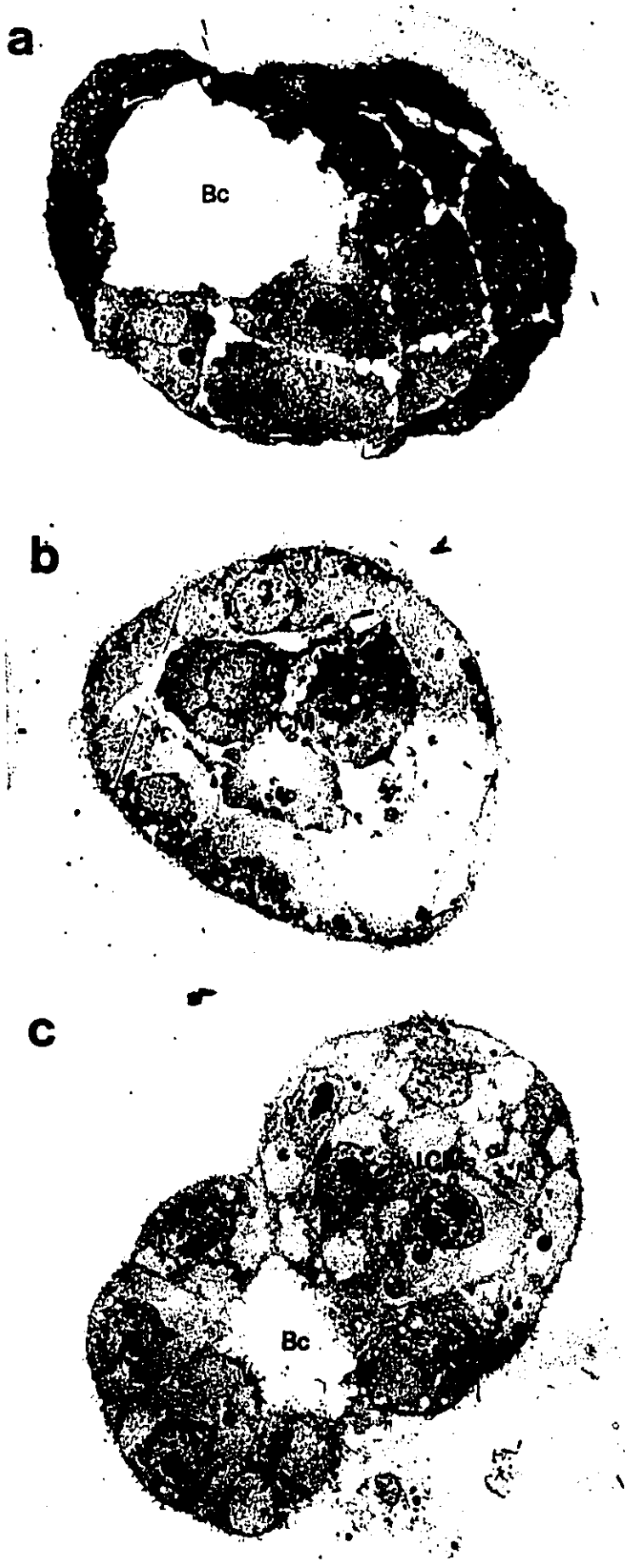

a
Bc
b
c
Bc

Figure 13

Electron micrographs demonstrating cytoplasmic organization of the three phenotypes of day 4.0 embryos sired by control males. The progressive segregation of cytokeratin arrays (C) and clustering of the organelles around the nucleus (N) was observed in these three phenotypes (first (a), second (b), and third (c)). The organelles included autophagic vesicles (filled arrows), Golgi apparatus (g), mitochondria (m), and occasional lipid vacuoles (L). Desmosomes (arrowheads) are present at cell contact points. Magnification 6100x.

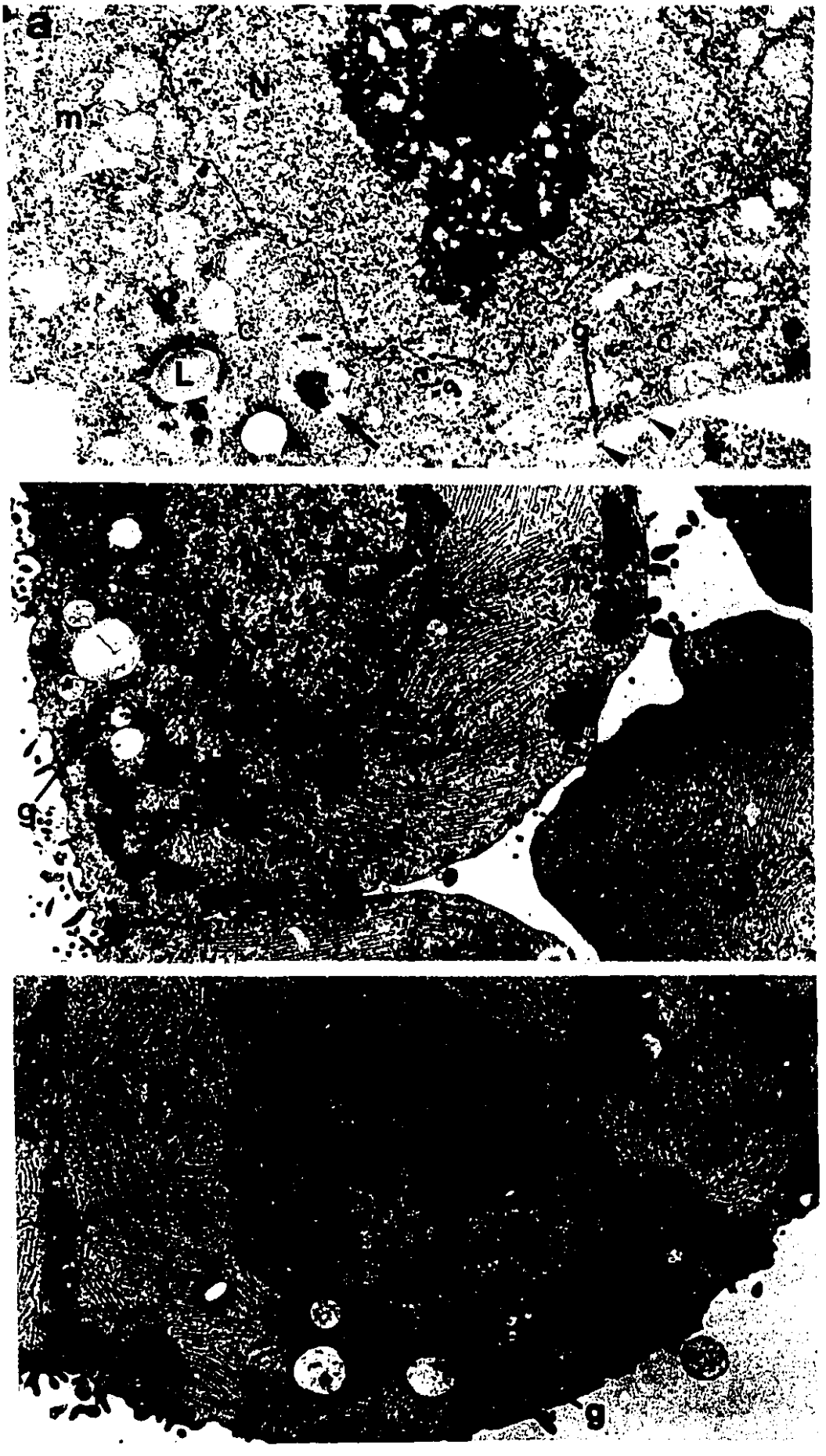

a
N
m
L
g
g
g

Figure 14

Electron micrographs demonstrating organelle structures in the three phenotypes of day 4 embryos sired by control males. The mitochondria (m) were transformed from spherical with a light matrix and marginal cristae in the first phenotype (a), to oval with a very dark matrix and both lamelliform and villous cristae in the section phenotype (b), to elongated with a medium-density matrix and perpendicular lamellar cristae in the third phenotype (c). Large autophagic vesicles (AV), rough endoplasmic reticulum (RER), and ribosomes progressively clustered around the nucleus (N), segregated from the cytokeratin arrays (C). Magnification 17700x.

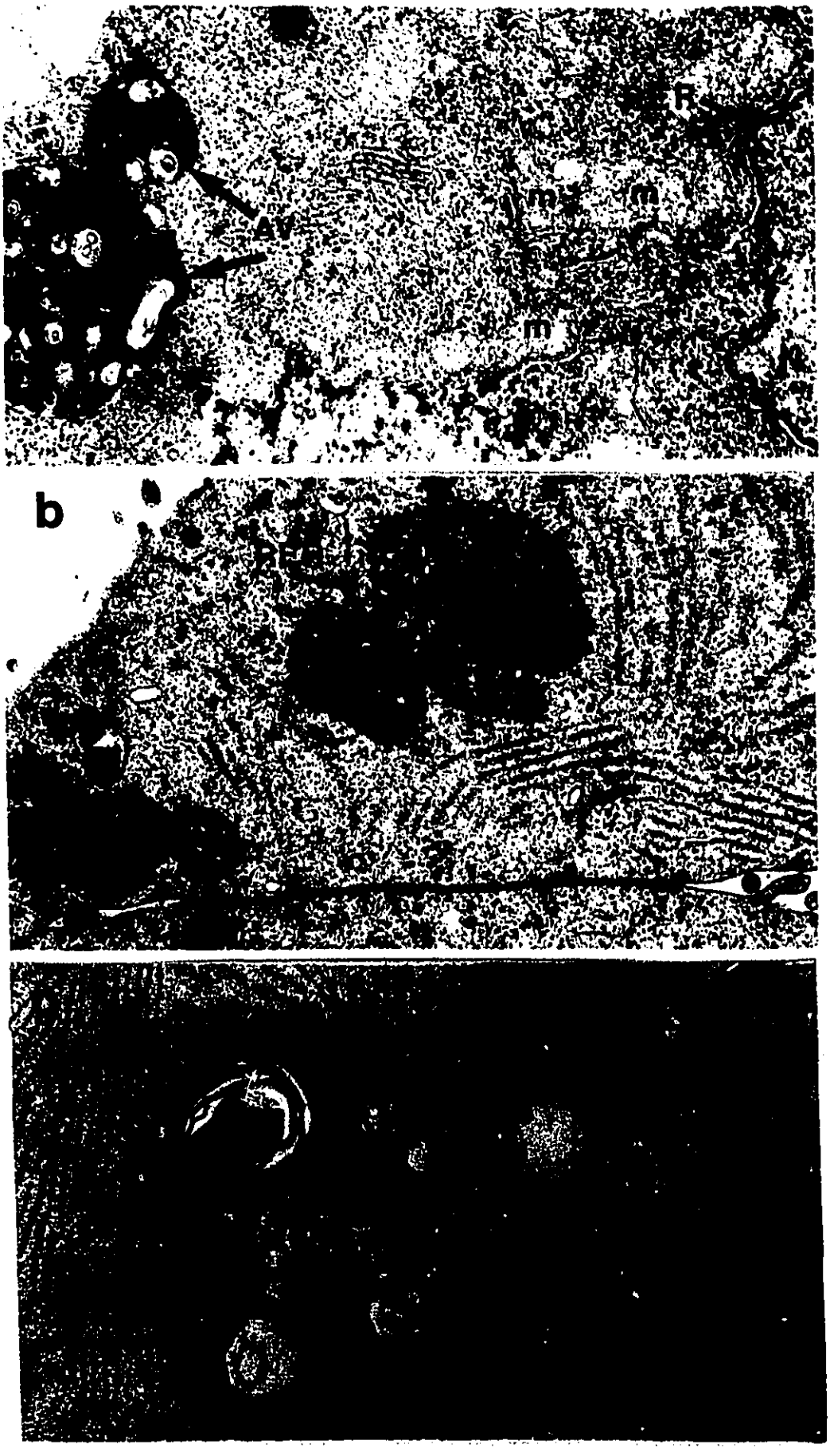

AV
R
m
m
m
b

Figure 15

Low magnification electron micrographs demonstrating the lack of trophectoderm ring formation in three day 4.0 embryos sired by two different cyclophosphamide-treated males. Variable cytoplasmic density was observed among the cells of the individual embryos (a,b,c). None of these three embryos had a recognizable continuous outer ring of trophectoderm cells (a,b,c). Zona pellucida (z); magnification 900x.

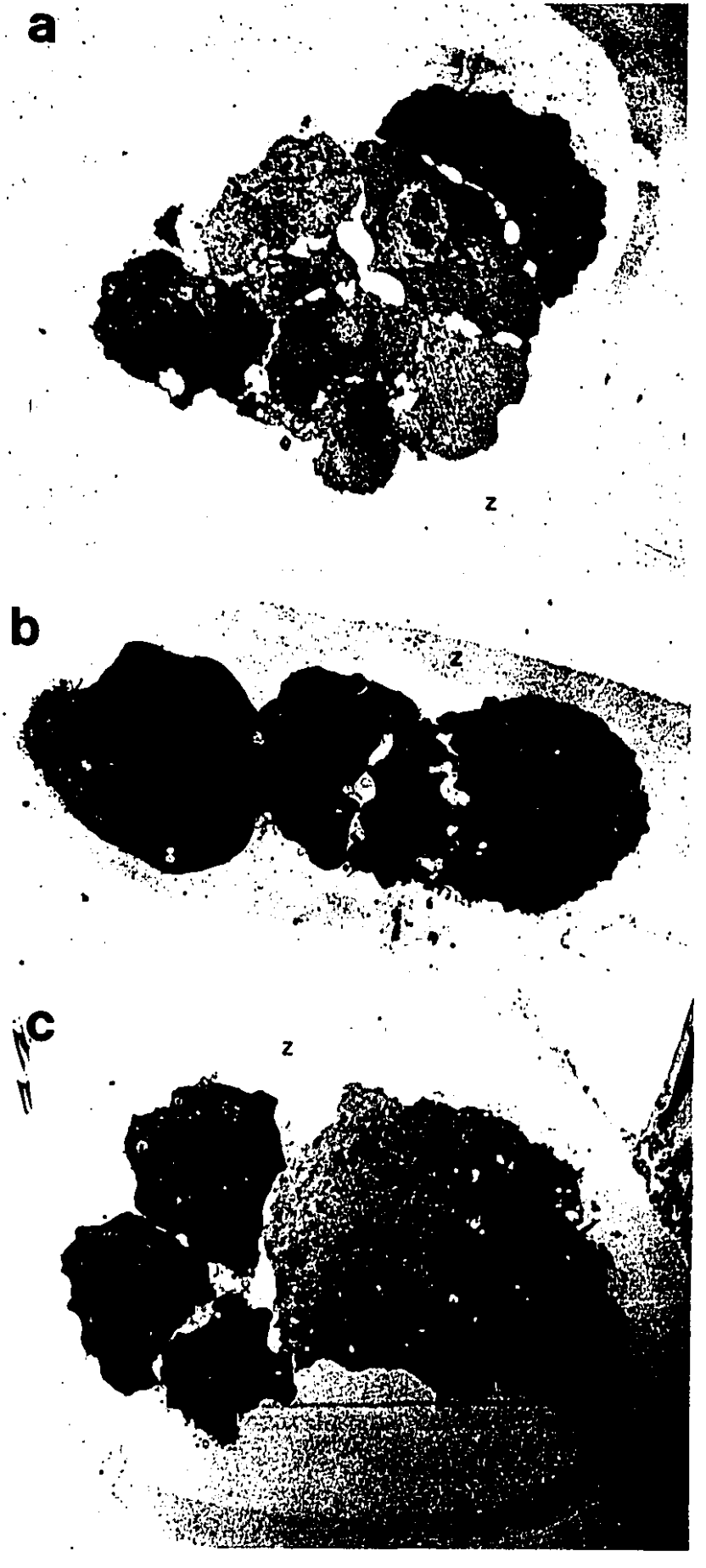

a
z
b
z
c
z

Figure 16

Electron micrographs demonstrating cytoplasmic organization in the three day 4.0 embryos, sired by cyclophosphamide-treated males, lacking a trophectoderm ring (a,b,c). Some desmosomes were present at cell contact points (arrowheads). Cells contained numerous small autophagic vesicles (filled arrows), cytokeratin arrays (C), mitochondria (m) of varying matrix density, and regions of smooth endoplasmic reticulum (sER). Note the small bodies containing chromatin (open arrows) separate from the larger nuclear structures (N) in two of these cells (a,c). Magnification 6100x.

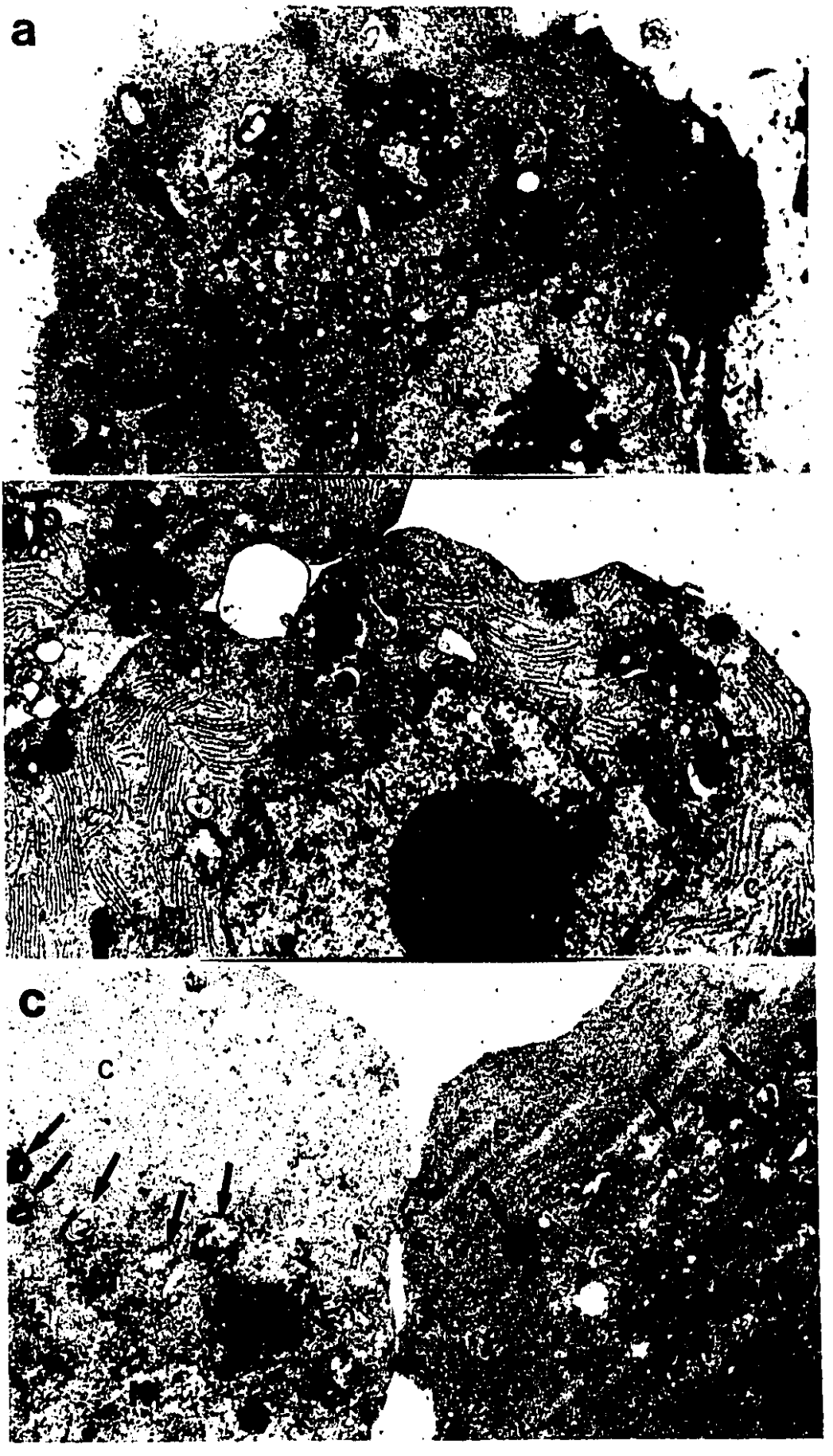

a
b
c
c

Figure 17

Electron micrographs demonstrating organelle structure in the three day 4.0 embryos, sired by cyclophosphamide-treated males, lacking a trophectoderm ring. At cell contact points, desmosomes (small arrowheads) were observed (a). Loops (small filled arrows) protruding from the cell membrane were also common (a,b). Large numbers of autophagic vacuoles containing myelin whorls and small vesicles (large arrowheads) or containing ribosomes (large filled arrows) were present in some cells (c). In other cells, swollen mitochondria (m) were observed (d). Cytokeratin arrays (C); magnification 17700x.

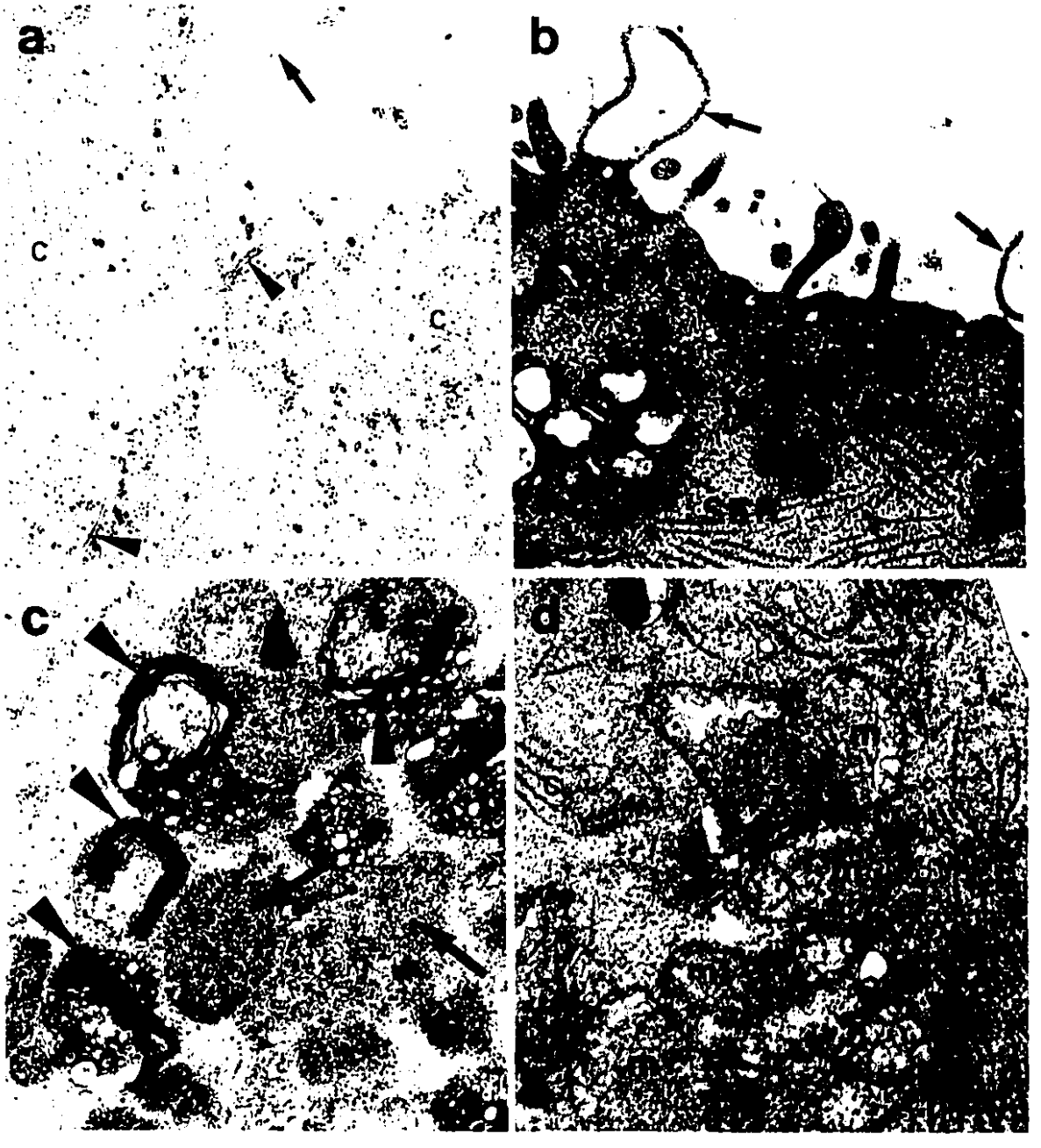

a
C
C
b
c
d

Figure 18

Low magnification electron micrographs of representative day 4.0 embryos, with trophectoderm rings, sired by two different cyclophosphamide-treated males. The zona pellucida (z) enclosed embryos (a,b) with a continuous, sealed trophectoderm surrounding inner cell mass cells (ICM) and blastocoel cavity (Bc). The blastocoel cavity is only partially visible in the section of the embryo shown in (a). Magnification 900x.

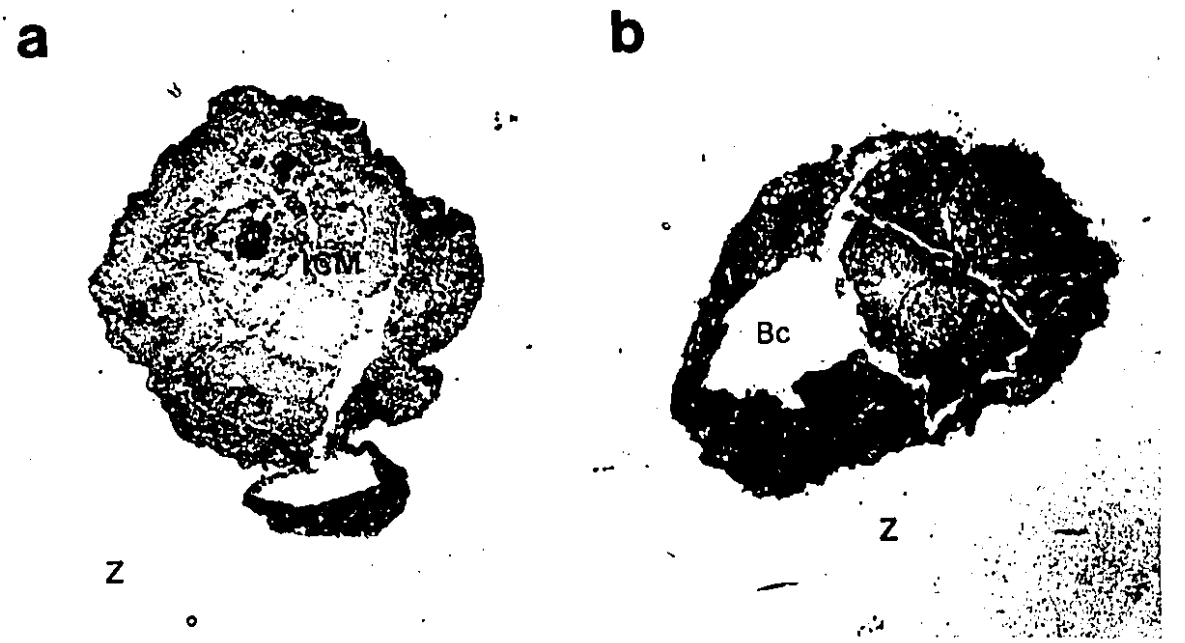

a
IgM
Z
b
Bc
Z

Figure 19

Electron micrographs demonstrating cytoplasmic organization of representative day 4.0 embryos with trophectoderm rings sired by two different cyclophosphamide-treated males. There was no segregation of cytoplasmic arrays (C) from other organelles. Mitochondria (m) were round with a light matrix. Numerous large autophagic vesicles (filled arrows) were observed; in one embryo (c), autophagic vesicles were packed with ribosomes. Nucleus, (n); magnification 6100x.

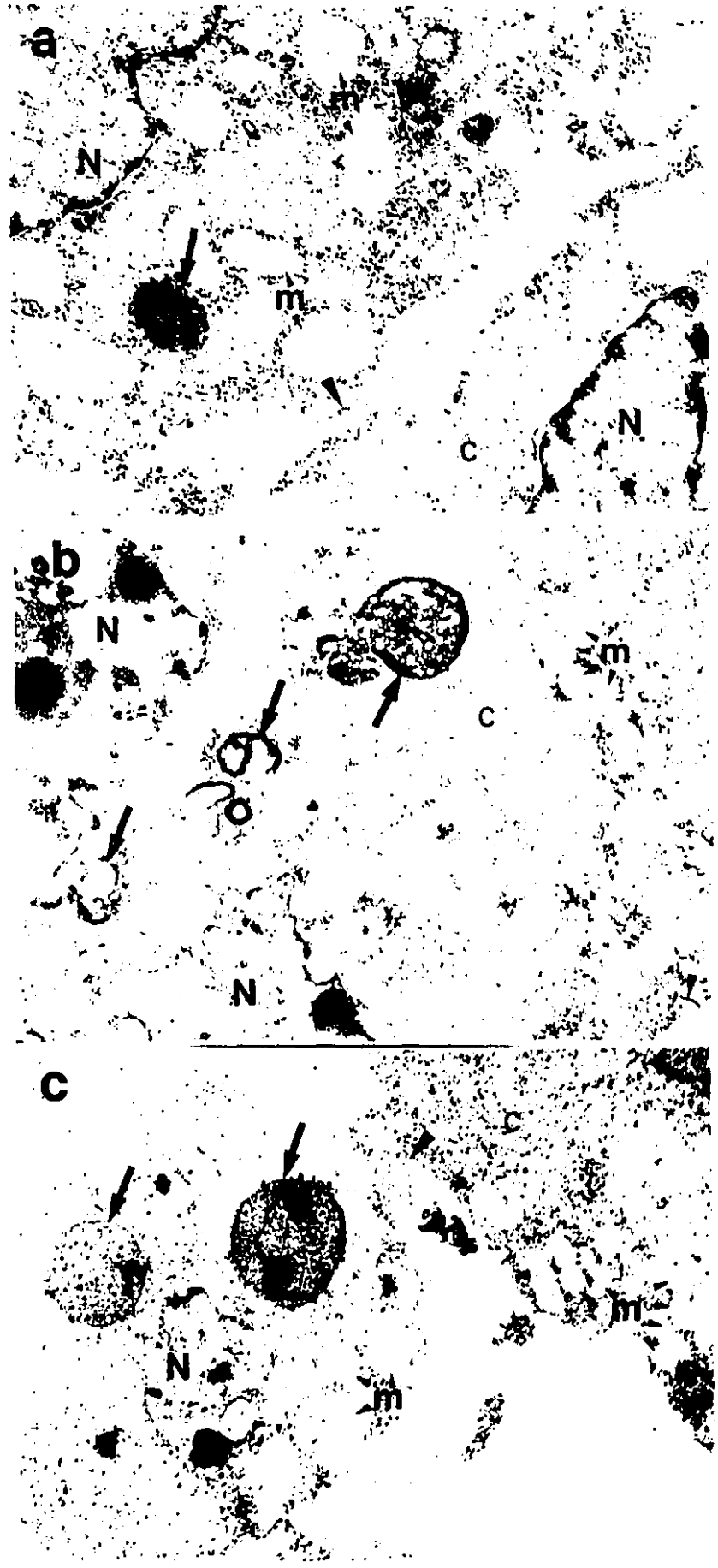
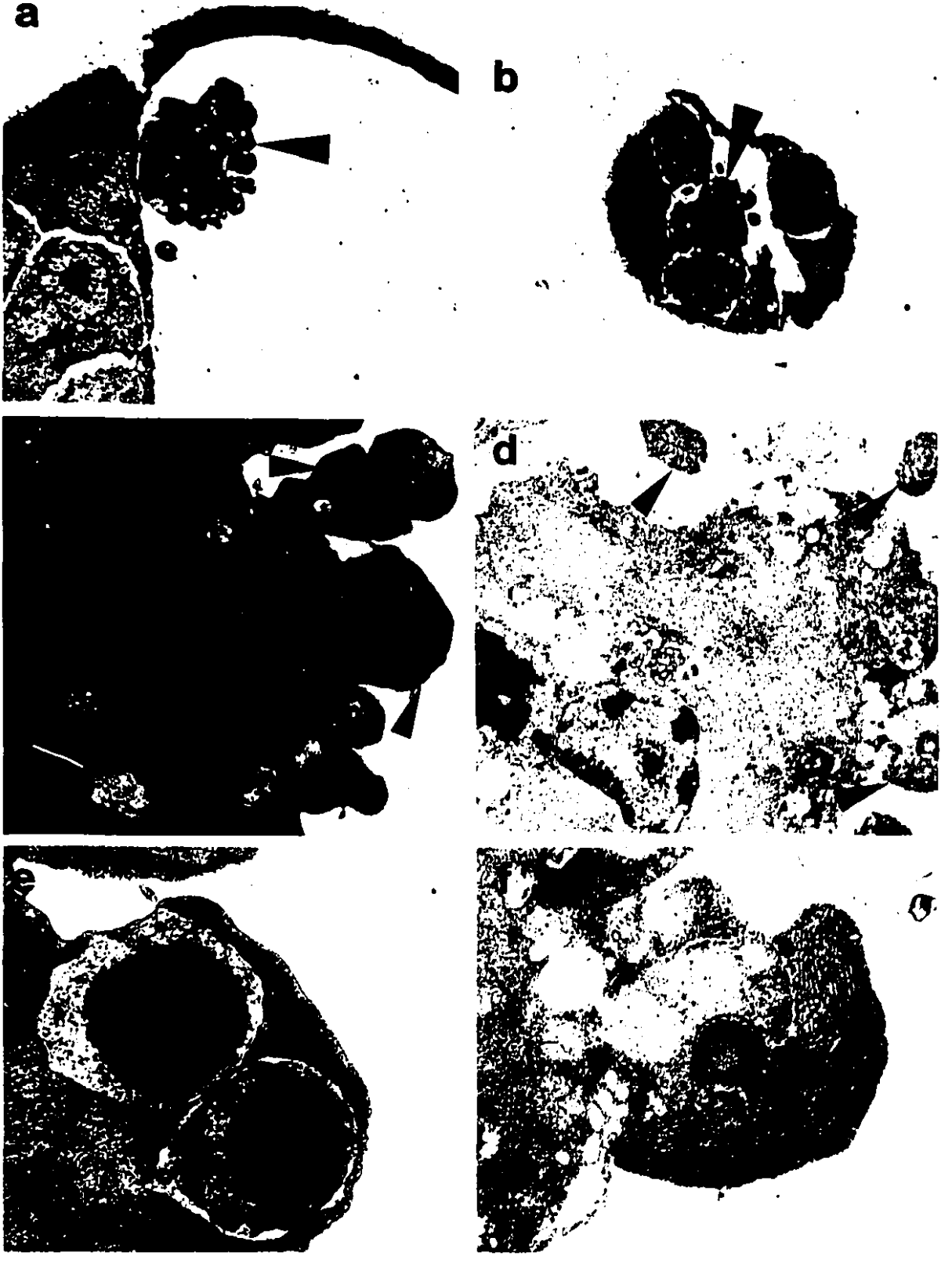

a
N
m
m
N
C
b
N
C
m
N
c
C
m
N
m

Figure 20

Apoptotic cells in day 4.0 embryos sired by control (a,c,e) and cyclophosphamide-treated (b,d,e) males. The apoptotic cells (arrowheads) were located inside the trophectoderm ring of the blastocyst (a,b). In both cases, bud-like projections (arrowheads) from the cell surface (c,d) contained nuclear and cytoplasmic fragments (e,f). Magnification (a,b): 900x; (c,d): 6100x; (e,f) 17700x.


a
b
d

# Chapter V

# Discussion

The studies in this thesis provide evidence that paternal exposure of the rat to the anticancer drug cyclophosphamide can affect the preimplantation development of his progeny. Embryos sired by cyclophosphamide-treated males have decreased rates of cell proliferation, resulting in depleted cell numbers at the blastocyst stage. The embryos implant into the uterine epithelium, but by day 7 of gestation the majority have little or no embryonic tissue and are subsequently resorbed. Despite the dramatic effect on cell proliferation, the critical morphological changes in early embryogenesis, including compaction, differentiation of inner cell mass and trophectoderm, and cavitation, appear unaffected. That drug exposure of the male germ cell prior to fertilization could have such dramatic effects on cell division in the embryo without affecting these important morphogenetic transitions leads to speculation about the specificity of cyclophosphamide damage to the paternal genome and the role of the paternal contribution in preimplantation embryogenesis.

This chapter will integrate the findings of this thesis and their implications into a putative model of germ cell damage and embryonic failure. First I will summarize the findings of this thesis work. In the second section I will discuss possible mechanisms of action of cyclophosphamide through the father in the light of the observed effects,

both at the level of the male germ cell and at the level of the developing embryo. Thirdly, I will speculate on a chronological scheme of development of those embryos sired by cyclophosphamide-treated males which are unable to develop normally. In the final section I will outline some of the questions for further investigation arising from this body of work.

A. Summary of Experimental Findings

Male rats treated for a four-week period with 6 mg/kg/day of cyclophosphamide had spermatozoa capable of fertilizing ova. The pregnant females which had been mated to treated males had implantation sites in their uteri on day 7 of gestation, containing some non-maternal cells and surrounded by a decidual reaction (Chap. 2). On day 7 of gestation there was a range in the degree of negative effects on the inner cell mass lineage of embryonic cells, ranging from severe retardation to complete absence.

Not only did paternal exposure to cyclophosphamide affect the morphology of the embryo on day 7, but deleterious effects could be observed during preimplantation development. The number of cells in embryos on day 2 and day 2.5 of gestation were not significantly different (Chap. 3). In most embryos from both groups the first cleavage from one cell to two-cell embryo occurred by 8-9 am on day 2 of gestation. Over the 12-hour period between day 2.0 and

day 2.5 the mean cell number increased to between 3 and 4 in both cases. Thus at least one blastomere and in most cases both blastomeres underwent another cleavage in the embryos in both groups.

Ultrastructural analysis revealed that in a small proportion of the embryos sired by treated males there was loss of membrane integrity of one blastomere while the others remained intact (Chap. 4). However, the subcellular appearance of the majority of the embryos sired by treated males was not dramatically different from controls.

Another cleavage of blastomeres occurred in both groups of embryos between day 2.5 and day 3.0 of gestation (Chap. 3). In the control group the mean cell number on day 3 was almost triple that on day 2.5. In the embryos sired by treated males, however, the cell number did not quite double from day 2.5 to day 3. Thus the first significant decrease in cell numbers occurred on day 3 of gestation.

Despite some litter-specific variability in morphology, there was no ultrastructural evidence of cell death accompanying the decrease in cell number on day 3 (Chap.4). Indeed, the embryos sired by cyclophosphamide-treated males were compacted like their control counterparts on day 3 of gestation (Chap. 4). Thus the damage caused by paternal cyclophosphamide exposure appeared to decrease cell number by decreasing the growth of the embryo rather than causing the death of embryonic cells.

Between day 3 and day 4 of gestation, embryos in both groups underwent further morphological transformations (Chap. 4). The inner cell mass and trophectoderm cell lineages differentiated. Blastocoele cavity formation began in embryos with enough cells to form an outer ring. Embryos isolated at 10 am on day 4 were in the midst of the blastocyst-stage change in cytoplasmic organization, in which there was segregation of cytokeratin sheet arrays from the other organelles, and transformation of mitochondrial morphology. In the embryos sired by cyclophosphamide-treated males, there was a subpopulation of embryos which did not have a continuous, sealed trophectoderm ring and did not undergo this transformation; the rest of the embryos from the treated group were at the same transition point as the controls. Thus, despite a drastic decrease in cell number in the treated group, the change in subcellular morphology was proceeding according to a normal developmental time frame, parallel to controls. This indicates that the changes in the paternal contribution resulting from cyclophosphamide exposure did not affect genes important for the progression of preimplantation morphogenesis.

In addition, during the day 3 to day 4 period there was a decrease in the amount of DNA synthesis in vitro in the embryos sired by cyclophosphamide-treated males compared to controls (Chap. 3). This decrease was reflected in changes

in cell number. In control embryos the number of cells almost tripled between day 3 and day 4, whereas in embryos sired by cyclophosphamide-treated males, the number on day 4 only slightly more than doubled. After day 4 of gestation, the amount of DNA synthesis per cell was the same in embryos sired by treated and control males. This would seem to indicate that the cells in embryos sired by treated males recovered from their initial dysfunction and were functioning at the proper DNA synthetic level. That the amount of DNA synthesis was decreased on a per cell basis between day 3 and day 4 indicated that the embryo may not yet have recovered from primary defects affecting proliferation.

Thus both the morphological appearance and normal DNA synthetic ability of the embryos sired by cyclophosphamide-treated males on day 4 of gestation indicate that these embryos were able to respond to morphogenetic cues and were capable of producing the proteins necessary to complete such transformations.

By day 4.5 of gestation the embryos sired by control males differentiated into distinct inner cell mass and trophectoderm lineages (Chap. 2). The embryos sired by cyclophosphamide-treated males had parallel decreases in inner cells and trophectoderm cells. Since the DNA synthesis per cell was the same in embryos sired by treated males as controls, the decrease in cell number must occur

prior to this differentiation process. In such a case the apportioning of cells to the two lineages would occur normally, but would be limited by the decreased cell number available in the starting material.

The presence of paternal chromosomes in embryos sired by treated males (Chap. 3) clarifies that paternal chromosomes are not destroyed by cyclophosphamide exposure but are capable of condensing and decondensing in dividing cells.

Paternal cyclophosphamide exposure causes a slowing of *in vitro* sperm nuclear decondensation (Qiu et al., personal communication). The present experiments do not measure whether *in vivo* decondensation on day 0.5 to day 1.0 of gestation is affected in embryos sired by cyclophosphamide-treated males. Such problems, if they occur, are likely to be due to protamine crosslinking rather than to damage to the sperm DNA itself. Protamines themselves are exchanged for histones almost immediately in the embryo (Nonchev & Tsanev, '90) and thus would not persist beyond the pronuclear stage.

There appear to be two critical moments in the development of embryos sired by cyclophosphamide-treated males. One occurs early during the preimplantation development of the embryo - there is a failure of some paternal growth signal at day 2.5 and the embryo falls behind in cell division. The second crisis occurs between

day 4 at 10 am and day 7 of gestation, when the inner cell mass-derived embryonic cells die (Chap. 2). Although the embryos seem to implant, indicating that trophectoderm cells were able to initiate hatching and invasion at the correct developmental time, something happens to retard or destroy the inner cell mass cells, followed by the death of the trophectoderm cells after day 7.

B. Peri-implantation Embryonic Failure - Possible Mechanisms

1. *Mechanisms of cyclophosphamide action at the level of the developing sperm*

The exposure of male rats to cyclophosphamide for a four-week period damages the germ cells such that most progeny die around implantation. What form does this damage take to cause specifically peri-implantation embryo death in the majority, but not all, fertilized embryos?

One recently proposed mechanism to explain the effects of very low doses of radiation on preimplantation embryo development is damage to the membrane of the spermatozoon (Wiley, '90). In such a case the damage would be transferred to the embryonic membrane, since elements of the sperm membrane persist until the blastocyst stage (Menge & Fleming, '78). Such a mechanism, solely involving membrane binding, is unlikely in the present model because transmissible heritable defects are observed in the $F_2$ generation after four weeks of treatment at a lesser dose

(5.1 mg/kg/day; Hales et al.,'92) - this is clear evidence of changes at the level of the DNA.

Changes in nuclear proteins could affect the genetic material indirectly. Cyclophosphamide metabolites are bifunctional, capable of alkylating and crosslinking proteins, particularly on sulfhydryl groups. The DNA of the maturing spermatid is closely packed into its condensed morphology in association with protamines, sperm proteins similar to histones which are specialized for this tight packing function (Balhorn, '82). Protamines are highly basic proteins containing numerous sulfhydryl groups, which gradually become oxidized as the spermatozoa travel through the epididymis (Shalgi et al., '89). Cyclophosphamide exposure for 6 weeks at 6 mg/kg/day decreases the number of free sulfhydryl and disulfide-linked groups in epididymal spermatozoa (Qiu et al., manuscript in preparation), presumably by alkylation. That similar treatment results in a significant increase in postimplantation loss (Trasler et al., '87) suggests that protamines could be important in the action of cyclophosphamide on the male germ cell. For some chemical mutagens, DNA breakage would seem to correlate more with protamine binding than with binding to DNA (Sega, '90). It is not clear how such alterations could result in changes in embryonic development.

One possibility is that altered protamines could affect the epigenetic alterations which lead to the imprinting of

paternal genes required for normal preimplantation development. Although the nature of such epigenetic signals is not clear, the phenomenon of parental imprinting can be observed in cases of both transgenes and endogenous genes (Monk & Surani, '90). For such a mechanism to be correct, the existence of some paternally-imprinted gene(s) required for a normal rate of preimplantation development and for peri-implantation survival would be presumed. The likelihood of such an imprinted paternal gene will be discussed with the mechanisms occurring at the level of the embryo in the next section.

Another possibility is that cyclophosphamide-mediated cross-linking of protamines could interfere with the decondensation of the sperm pronucleus in the egg. A six-week treatment with 6 mg/kg/day cyclophosphamide causes a significant decrease in the rate of in vitro decondensation of sperm nuclei (Qiu et al., manuscript in preparation). If the rate of pronuclear decondensation is slowed in vivo, there may be problems with DNA replication and cleavage. If the protamines are chemically cross-linked, the normal exchange of protamines for histones in the zygote (Nonchev & Tsanev, '90) could be delayed, and could potentially interfere with the cell cycle and the timely activation of embryonic genes.

Cyclophosphamide metabolites can directly alkylate DNA, and this remains the most likely mechanism of action.

Mutations resulting from alkylation of deoxynucleotide bases or as a result of cross-linking of DNA strands are not repaired in the post-meiotic germ cell (Sotomayor & Cumming, '74).

The common result of DNA damage would be an array of random mutations, different in each individual sperm. Such mutations would have to cause enough generalized damage to the paternal genome that in most cases there would be damage to a gene required for the development of the late blastocyst. The damage caused by chronic 4-week treatment with 6 mg/kg/day cyclophosphamide is not so severe as to cause drastic chromosome breakage, because cells of embryos sired by treated males are able to divide and at the blastocyst stage still contain mitotic figures with 42 chromosomes (Chap. 3). It is not known how many genes are active during preimplantation development, or how many are coded from the paternal genome, but the number of new proteins presumably synthesized from embryonic transcripts is low (Kidder '92). Certainly some gene transcription and protein synthesis is required for normal preimplantation development (Kidder & McLaughlin, '85), but haploid gynogenetic embryos, completely lacking a paternal genome, are able to divide slowly through preimplantation development and do not die until the peri-implantation stage (Henery & Kaufman, '92). It is conceivable, then, that the paternal genome is not absolutely required for

preimplantation development, although the process is slowed if it is lacking - but is needed when the large burst of transcription and translation activity occurs at gastrulation.

There has been speculation that certain chromosomes are more vulnerable to cyclophosphamide damage than others (Morad & El Zawahri, '77). If such specificity exists it is more likely a characteristic of the target than a characteristic of the alkylating agent. The selective vulnerability of certain regions of DNA could be due to the organization of the chromatin rather than any specificity of cyclophosphamide action on the male genome. There are regions of chromatin in the condensing spermatid nucleus where restriction enzymes are able to cut far more easily than surrounding regions (McPherson & Longo, '92). These regions of "active chromatin" are the same in each spermatid and are hypothesized to contain genes still being transcribed which are important for the ongoing spermiogenic process (Elgin, '88). Such an "open" conformation could also render such sites preferentially vulnerable to damage by alkylating agents. A developmentally-important gene located near or within such an "active chromatin" region could sustain damage by its proximity.

It is important to remember that more than one of these mechanisms of action may act concurrently. As described above, a number of different possible mechanisms of

cyclophosphamide damage can lead to the same final target - the DNA. That some transmissible genetic damage is present in the germ cells of the surviving $F_1$ generation is clear (Hales et al., '92). Whether a similar mechanism of cyclophosphamide action results in peri-implantation death of their littermates cannot yet be concluded.

The survival of 10% of the offspring of cyclophosphamide-treated males would indicate that the critical damage causing death of siblings at the peri-implantation stage does not occur in these embryos. One explanation of these results is that a dose of 6 mg/kg/day of cyclophosphamide for 4 weeks is not able to saturate all sites vulnerable to alkylation in the paternal genome, but would seem to miss the "preimplantation-critical site(s)" 10% of the time. The effect of lower doses of cyclophosphamide on postimplantation loss is proportionally less severe (Trasler et al., '86), implying that the frequency of critical damage depends on the concentration of cyclophosphamide. Alternatively, the 10% of embryos sired by cyclophosphamide-treated males which survive to birth could represent embryos which are not affected severely enough to decrease their cell number at the preimplantation stage below the critical minimum. The damage they sustain to their paternal complement does not kill them, but, in combination with other offspring of treated males, their inherited mutations cause 47% postimplantation loss in the

$F_2$ generation (Hales et al., '92). It has been suggested that balanced translocations occurring in the $F_1$ generation could result in unbalanced segregants in the subsequent generation (Hales et al., '92).

2. *Mechanisms of dysfunction at the level of the embryo*

Genes coding for structurally or functionally-important proteins could be mutated in the paternal genome during cyclophosphamide exposure. It is possible that embryo failure could result from the inappropriate expression of a normal gene product or the expression of a mutant protein. This is not necessarily restricted to a lack of protein - the loss of noncoding genetic sequences required for the activation of important genes could also have drastic effects on embryo development.

It is possible that the delay in preimplantation development could have been due to a mutation which did not prevent DNA replication but changed the ability of the embryonic genome to interact with and regulate itself. For instance, damage to a gene encoding a transcription factor which activates developmentally-important genes, or to the enhancer, response element or promoter regions of a developmentally-important gene could result in such effects. The embryos sired by treated males did not undergo a cessation of development but appeared to briefly slow their rate of cell proliferation, then recover normal

developmental speed at reduced cell numbers. The absence of significant preimplantation loss after 4 weeks of treatment (Trasler et al., '87) would seem to indicate that the manifestation of cyclophosphamide damage in the embryo is transient and not cell-lethal. A dose-effect argument could be invoked in which the embryo requires both parental alleles of a given gene in order to progress at a regular developmental speed. Embryos sired by treated males with a defect in the paternal allele of such a gene would delay development while sufficient product was made from the maternal allele. Such a lack of a strategic paternal allele would also explain the delayed preimplantation growth of the haploid embryo (Henery & Kaufman, '92).

A number of paternal genes must code for housekeeping proteins necessary for ongoing cell survival. Proteins involved in cell interaction, cavitation, differentiation of inner cell mass and trophectoderm, and reorganization of cytoplasmic structures are coded from embryonic genes (Kidder, '92; Schultz & Heyner, '92). Surely if paternal DNA was extensively damaged, some of these genes would be affected. It may be that the paternal genome is simply strangely unnecessary for preimplantation development. The ability of diploid embryos completely lacking paternal genes to exhibit a normal rate of preimplantation growth (Henery & Kaufman, '92) supports the idea that no imprinted paternal genes are required for preimplantation growth.

In mouse embryos homozygous for the lethal yellow allele at the agouti locus ($A^{Y}/A^{Y}$), embryos fail to develop past the peri-implantation stage of development (Pedersen, '74). In these embryos the DNA is rearranged such that the *Raly* gene which codes for an RNA-binding protein is disrupted, and the agouti gene loses its control elements (Michaud et al., '93). Because of this the agouti product is ectopically expressed throughout embryogenesis, and the product of the *Raly* gene normally present at the blastocyst stage is not expressed (Michaud et al., '93). Since in heterozygous form the $A^{Y}$ allele would still ectopically express agouti, the authors propose that it is the lack of *Raly* protein at the blastocyst stage which causes embryolethality (Michaud et al., '93). This provides evidence that a lack of protein due to gene mutation could result in retarded preimplantation development and embryo death.

The albino deletions in the mouse have been extensively studied (Gluecksohn-Waelsch '79) and have revealed that the albino locus, a region on chromosome 7, is important for embryo development. When homozygous these deletions result in embryonic death at varying stages of development from the preimplantation stage (eg. $c^{25H}/c^{25H}$) to the perinatal stage (eg. $c^{6H}/c^{6H}$). The phenotypes of heterozygous and homozygous embryos strongly suggest that the deletions include a gene or genes controlling or regulating another specific set of

structural genes located on other chromosomes (Gluecksohn-Waelsch '79).

There are several other examples of single mutation sites where homozygosity results in a phenotype similar to that observed in the present study. Tail-short ($T^s$) (Paterson, '80), hairpin ($T^{hp}$), and orleans ($T^{Orl}$)(Babiarz,'83) at the *t*-locus, lethal nonagouti ($A^x$) (Papaioannou & Mardon, '83), and the insertional transgenic mutation rß3 (Mark et al., '85) are all single-locus mutations which result in retarded preimplantation development and peri-implantation embryonic death. Clearly there are a number of single genes required for preimplantation development.

It is important to note, however, that the embryolethal damage to the paternal genome incurred by chronic cyclophosphamide treatment acts in a dominant lethal manner. A dominant lethal mutation refers to embryonic death resulting from chromosome breakage in the germ cells of only one parent (Ehling, '77). A number of mechanisms have been proposed for such breakage, including cross-linking of DNA strands preventing DNA replication in the zygote, double-strand breaks, and depurinations (Ehling et al., '68). Cyclophosphamide induces chromosome aberrations (Rathenberg & Müller, '72; Pacchierotti et al., '83; Jenkinson & Anderson, '90), translocations (van Buul, '84), and micronucleus formation (Lähdetie, '88) in male germ cells.

Cyclophosphamide could cause double strand breaks resulting in partial or complete loss of chromosomes in the paternal genome. The observed chromosome counts varying from 36.5 - 42 do not rule out the possibility of chromosomal aberrations in the embryos sired by cyclophosphamide-treated males. Thus comparison with conditions of chromosome imbalance such as nullisomy, monosomy, and haploidy where the presence of a single chromosome is insufficient for proper embryo development may be more relevant.

Autosomal monosomy in the mouse results in peri-implantation embryonic death. Monosomic embryos die by the early blastocyst stage (monosomies 1, 2, 3, 5, 6, 11, 15, and 16), the late blastocyst stage (monosomies 14 and 19), or at early postimplantation stages (monosomies 4, 10, 12, 17, and 18) (Baranov, '83; Magnuson et al., '85). Mouse embryos entirely lacking the paternal chromosomal complement (parthenogenetic haploid) have significantly decreased cell numbers during preimplantation development (Henery & Kaufman, '92) and very little if any postimplantation development (Kaufman, '78). It is interesting that the loss of a single chromosome in early blastocyst-lethal autosomal monosomies has more severe effects than the loss of an entire haploid chromosome set.

The decreased proliferation of preimplantation haploid parthenogenetic embryos appears to be due to a lack of diploidy rather than some unique paternal requirement from

the male genome, since diploid parthenogenetic embryos containing two maternal pronuclei develop at a normal rate (Henery & Kaufman, '92). In fact, it appears that the paternal genome is not absolutely required until the peri-implantation period, when diploid parthenogenetic embryos are unable to develop extraembryonic tissues normally (Kaufman et al., '77). Since androgenetic diploid embryos only form apparently normal blastocysts about 20% of the time (Surani, '86), it would appear that although diploidy is required, the maternal genome carries additional unique genes required for preimplantation development. If a gene important for proliferation, requiring transcription from both parental alleles, is damaged at the paternal allele, the embryo may slow down until maternal product compensates.

There seems to be a lack of correlation between preimplantation gene expression and the requirement for those proteins necessary for morphogenetic processes such as compaction, cavitation, and blastocoel expansion (Kidder, '92). Indeed, the number of cells present in the embryo has no effect on the formation of the blastocoele cavity in the mouse embryo (Smith & McLaren, '77). Thus, it may not be surprising that the embryos sired by cyclophosphamide-treated males undergo all of these morphogenetic changes, even when only half the number of cells are present.

In many cases the inner cell mass lineage is more vulnerable to the deleterious effects of exogenous agents

than the trophectoderm cells (Chap. 1). In the present model there is a nonspecific decrease in the number of cells of both lineages on day 4.5 of gestation. This somehow results in implantation sites on day 7 of gestation containing trophectoderm cells but very few or no inner cell mass cells. One explanation for the increased vulnerability of the inner cell mass cells is that they divide more rapidly than the trophectoderm cells. Giant trophoblast cells are in fact non-dividing and may thus be able to survive sharp reductions in the rate of DNA, RNA or protein synthesis (Sherman & Atienza, '75). However, there is no proof that the trophectoderm cells are unaffected in these implantation sites - they may cause the death of the inner cell mass cells by ceasing to provide the nutrients needed for growth in an attempt to rescue their own survival.

Trophectoderm cell proliferation has been suggested to depend upon a minimum number of inner cell mass cells (Ansell & Snow, '75). Mouse embryos treated with aphidicolin formed blastocysts containing half the normal number of cells; these formed implantation sites containing only trophectoderm cells (Dean & Rossant, '84). Thus the postimplantation failure of rat embryos sired by cyclophosphamide-treated males could be due simply to a deficit of inner cells.

C. From Spermatozoon to Conceptus - a Putative Model

Based on the present results, a speculative model of the action of cyclophosphamide on the germ cell and the resultant failure of the embryos can be postulated.

The spermatids in the testis receive the initial dose of cyclophosphamide as the metabolites phosphoramide mustard and acrolein. The DNA of the spermatid condenses in a specific pattern of chromatin packing in which sections of the DNA, the DNAse hypersensitive sites, are more vulnerable to the damage of alkylating agents. Both these hypersensitive sites and protamines are alkylated by phosphoramide mustard and acrolein. The continuing presence of cyclophosphamide metabolites in the seminiferous tubules ensures that most of the vulnerable sites are alkylated. One of these sites contains a specific gene, expressed at the day 2 to day 2.5 time point, which is necessary for embryonic genome activation. Ninety percent of the sperm have alterations at this site which result in the non-expression of this gene product in the embryo.

Further damage to the spermatozoa occurs in the epididymis (Qiu et al., '92). A number of sites, including the one containing the early expressing gene, remain vulnerable for alkylation in the maturing spermatozoa as it passes through the first half of the epididymis. The normal disulfide linkage of the protamine sulfhydryl groups is partially precluded by the presence of extensive alkyl moieties. Capacitation of spermatozoa is not prevented,

however, and the ejaculated spermatozoa are capable of fertilizing the ovum.

Fertilization progresses in a manner satisfactory for the production of a male pronucleus. There may be some slowing of the decondensation process and protamine exchange for histones in the egg, but this is insufficient of itself to retard the embryo lethally. Other vulnerable sites contain specific genes required in at least a haploid dose at development. These are mutated but do not affect the development of the surviving $F_1$ embryos. These mutations become consequential when homozygous in the $F_2$ generation, causing a dramatic increase in postimplantation loss.

The embryonic genome is activated at the two cell stage, at which point the paternal damage to the specific embryonic gene important for activation of the embryonic genome manifests itself. The difficulties in initiating transcription from the paternal genome cause a delay in the cell cycle. Sufficient transcription of the maternal allele eventually occurs for the progression of preimplantation development. This delay in the activation of the paternal genome causes the embryos to be retarded in their development, such that the proliferation of cells is slower at the day 3 time point compared to controls. By day 4 the embryos sired by cyclophosphamide-treated males return to normal speed of cell division, and are able to respond to cues not only decreasing cell cycle length but also

signalling morphogenetic changes. Compaction at the 4- to 8-cell stage (normally at the 8-cell stage, but the embryo is retarded by problems with DNA transcription) and cavitation on day 4 occur at the proper times because these are independent of cell number or the number of cell divisions (Smith & McLaren, '77; Dean & Rossant, '84).

Another surge in the activation of embryonic genes occurs in the mid- to late blastocyst stage when the inner cell mass and trophectoderm cells begin to differentiate. The embryo dies at implantation because there are insufficient inner cells to continue development (Ansell & Snow, '75). The trophectoderm cells may persist because they are directly in contact with uterine cells and may be more dependent on external stimulation and less on their own genetic activation.

## D. Integration of Present Studies and Future Directions

The results obtained in the present thesis provide a preliminary time line for the defective development of the embryos sired by cyclophosphamide-treated males. With such information it becomes possible to ask questions about the effects of cyclophosphamide-induced damage on specific embryonic functions.

Embryos sired by cyclophosphamide-treated males contain less than half the number of cells found in control embryos on day 4 of gestation. As described above, normal embryos

fail to thrive if they lack sufficient cell numbers (Ansell & Snow, '75). If this were the case for the progeny of cyclophosphamide-treated males, their development would be rescued by the simple aggregation of pairs of such embryos. Similarly, if embryos sired by treated males lack diffusible factors or contact with normal cell surfaces, their development should be rescued in chimeras with normal embryos. A lack of rescue would indicate that the defect in embryonic development was cell-autonomous. If fluorescent tracers were used to mark the cells of embryos sired by treated males, such experiments could also give useful information about the lineage-specificity of paternal cyclophosphamide-mediated defects. Recombinant blastocysts combining inner cell masses and trophectodermal vesicles of embryos sired by control and treated males would also clarify whether the death of the embryo is due to a defect in one cell lineage or in both.

Clearly on day 4 of gestation embryos sired by treated males contain less than one half the cells of their control counterparts (Chap. 3). Mouse embryos with less than one half the normal cell number are unable to hatch normally from the zona pellucida (Gordon et al., '93a, 93b). Between day 4 and day 7 a decidual reaction is induced by embryos sired by treated males, and embryonic cells enter the implantation site. Is there a delay in hatching function of the embryos sired by cyclophosphamide-treated males? Do

embryos begin to degenerate before, during, or after attachment to the uterine epithelium? The in vitro culture of day 4 embryos to observe their hatching and attachment would clarify the progress of embryonic dysfunction between day 4 and day 7.

Is there expression of a defective protein or a lack of a normally-expressed protein over the day 2.5 to day 5 period when cell numbers are decreasing? Comparative 2-D gel electrophoresis would give an indication of any gross qualitative or quantitative changes in proteins in preimplantation embryos sired by cyclophosphamide-treated males, and provide a starting point for the examination of specific genes which might be altered after cyclophosphamide exposure.

References

Ansell JD, Snow MHL. The development of trophoblast in vitro from blastocysts containing varying amounts of inner cell mass. J Embryol exp Morphol 1975; 33:177-185.

Babiarz BS. Deletion mapping of the *T/t* complex: evidence for a second region of critical embryonic genes. Dev Biol 1983; 95:342-351.

Balhorn R. A model for the structure of chromatin in mammalian sperm. J Cell Biol 1982; 93:298-305.

Baranov VS. Chromosomal control of early embryonic development in mice. I. Experiments on embryos with autosomal monosomy. Genetica 1983; 61:165-177.

Dean WL, Rossant J. Effect of delaying DNA replication on blastocyst formation in the mouse. Differentiation 1984; 26:134-137.

Ehling UH. Dominant lethal mutations in male mice. Arch Toxicol 1977; 38:1-11.

Ehling UH, Cumming RB, Malling HV. Induction of dominant lethal mutations by alkylating agents in male mice. Mutat Rese 1968; 5:417-428.

Elgin SCR. The formation and function of DNase I hypersensitive sites in the process fo gene activation. J Biol Chem 1988; 263:19259-19262.

Gleucksohn S. Genetic control of morphogenetic and

biochemical differentiation: lethal albino deletions in the mouse. Cell 1979; 16:225-237.

Gordon JW, Dapunt U. A new mouse model for embryos with a hatching deficiency and its use to elucidate the mechanism of blastocyst hatching. Fert Steril 1993; 59:1296-1301.

Gordon JW, Dapunt U. Resoration of normal implantation rates in mouse embryos with a hatching impairment by use of a new method of assisted hatching. Fert Steril 1993; 59:1302-1307.

Hales BF, Crosman K, Robaire B. Increased postimplantation loss and malformations among the $F_2$ progeny of male rats chronically treated with cyclophoshamide. Teratol 1992; 45:671-678.

Henery CC, Kaufman MH. Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. Mol Reprod Dev 1992; 31:258-263.

Jenkinson PC, Anderson D. Malformed foetuses and karyotype abnormalities in the offspring of cyclophosphamide and allyl alcohol-treated male rats. Mutat Res 1990; 229:173-184.

Kaufman MH. Chromosome analysis of early postimplantation presumptive haploid parthenogenetic mouse embryos. J Embryol exp Morphol 1978; 45:85-91.

Kaufman MH, Barton SC, Surani MAH. Normal postimplantation

development of mouse parthenogenetic embryos to the forelimb bud stage. Nature 1977; 265:53-55.

Kidder GM. The genetic program for preimplantation development. Dev Genet 1992; 13:319-325.

Kidder GM, McLaughlin JR. Timing of transcription and protein synthesis underlying morphogenesis in preimplantation mouse embryos. Dev Biol 1985; 112:265-275.

Lähdetie J. Induction and survival of micronuclei in rat spermatids. Comparison of two meiotic micronucleus techniques using cyclophosphamide. Mutat Res 1988; 203:47-53.

Magnuson T, Debrot S, Dimpfl J, Zweig A, Zamora T, Epstein CJ. The early lethality of autosomal monosomy in the mouse. J Exp Zool 1985; 236:353-360.

Mark WH, Signorelli K, Lacy E. An insertional mutation in a transgenic mouse line results in developmental arrest at day 5 of gestation. ColD Spring Harbor Symp Quant Biol 1985; 50:453-463.

McPherson SM, Longo FJ. Localization of DNase I-hypersensitive regions during rat spermatogenesis: stage-dependent patterns and unique sensitivity of elongating spermatids. Mol Reprod Dev 1992; 31:268-279.

Menge AC, Fleming CH. Detection of sperm antigens on mouse ova and early embryos. Dev Biol 1978; 63:111.

Michaud EJ, Bultman SJ, Stubbs LJ, Woychik Rp. The embryonic lethality of homozygous lethal yellow mice ($A^{Y}/A^{Y}$) is associated with the disruption of a novel RNA-binding protein. Genes Dev 1993; 7:1203-1213.

Monk M, Surani A, eds. Genomic Imprinting (Dev. Suppl.), The Company of Biologists, Ltd., Cambridge, United Kingdom, 1990.

Morad M, El Zawahri M. Non-random distribution of cyclophosphamide-induced chromosome breaks. Mutat Res 1977; 42:125-130.

Nonchev S, Tsanev R. Protamine-histone replacement and DNA replication in the male mouse pronucleus. Mol Reprod Dev 1990; 25:72-76.

Pacchierotti F, Bellincampi D, Civitareale D. Cytogenetic observations, in mouse secondary spermatocytes, on numerical and structural chromosome aberrations induced by cyclophosphamide in various stages of spermatogenesis. Mutat Res 1983; 119:177-83.

Papaioannou VE, Mardon H. Lethal nonagouti ($a^{x}$): description of a second embryonic lethal at the agouti locus. Dev Genet 1983; 4:21-29.

Paterson HF. In vivo and in vitro studies on the early embryonic lethal Tail-short (*Ts*) in the mouse. J Exp Zool 1980; 211:247-256.

Pedersen RA. Development of lethal yellow ($A^{Y}/A^{Y}$) mouse embryos in vitro. J Exp Zool 1974; 188:307-320.

Qiu J, Hales BF, Robaire B. Adverse effects of cyclophosphamide on progeny outcome can be mediated through post-testicular mechanisms in the rat. Biol Reprod 1992; 46:926-931.

Rathenberg R, Müller D. Comparative cytogenetic studies of the influence of phenylbutazone and cyclophosphamide on spermatogenesis in the mouse. Agents Actions 1972; 2:180-185.

Schultz GA, Heyner S. Gene expression in pre-implantation mammalian embryos. Mutat Res 1992; 296:17-31.

Sega GA. Molecular targets, DNA breakage and DNA repair: Their roles in mutation induction in mammalian germ cells. In: Allen JW, Bridges BA, Lyon MF, Moses MJ, Russell LB, (eds.). Banbury Report 34: Biology of Mammalian Germ Cell Mutagenesis. New York:Cold Spring Harbor Laboratory Press; 1990:79-91.

Shalgi R, Seligman J, Kosower. Dynamics of the thiol status of rat spermatozoa during maturation: analysis with the fluorescent labeling agent monobromobimane. Biol Reprod 1989; 40: 1037-1945.

Sherman MI, Atienza SB. Effects of bromodeoxyuridine, cytosine arabinoside and colcemid upon the in vitro development of mouse blastocysts. J Embryol exp Morphol 1975; 34:467-484.

Smith R, McLaren A. Factors affecting the time of formation of mouse blastocoele. J Embryol exp Morphol 1977;

41:79-92.

Sotomayor RE, Cumming RB. Induction of translocations by cyclophosphamide in different germ cell stages of male mice: cytological characterization and transmission. Mutat Res 1975; 27:375-388.

Surani MAH. Evidence and consequences of differences between maternal and paternal genomes during embryogenesis in the mouse. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian Embryonic Development. New York: Cambridge University Press; 1986: 401-435.

Trasler JM, Hales BF, Robaire B. Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. Biol Reprod 1986; 34:275-283.

Trasler JM, Hales BF, Robaire B. A time-course study of chronic paternal cyclophosphamide treatment in rats: effects on pregnancy outcome and the male reproductive and hematologic systems. Biol Reprod 1987; 37:317-326.

Van Buul PPW. Enhanced radiosensitivity for the induction of translocations in mouse stem cell spermatogonia following treatment with cyclophosphamide or adriamycin. Mutat Res 1984; 128:207-211.

Wiley LM. What is the radiosensitive target of mammalian gametes and embryos at low doses of radiation? In: Allen JW, Bridges BA, Lyon MF, Moses MJ, Russell LB,

(eds.). Banbury Report 34: Biology of Mammalian Germ Cell Mutagenesis. New York:Cold Spring Harbor Laboratory Press; 1990:299-310.

## List of Original Contributions

1. Male rats were given daily doses of 6 mg/kg/day of cyclophosphamide for 4 weeks, then mated to untreated females. By examination of histological sections of implantation sites isolated on day 7 of gestation, I showed for the first time a specific retardation or absence of inner cell mass-derived embryonic cells after paternal drug treatment.

2. I characterized the beginning of abnormal development of embryos sired by cyclophosphamide-treated males as occurring at preimplantation stages; significant reductions in cell numbers were observed as early as day 3 (37% decrease) and day 4 (55% decrease) of gestation. Cell numbers were not significantly decreased in embryos sired by treated males on day 2 or day 2.5 of gestation.

3. I showed that embryos sired by cyclophosphamide-treated males had decreased DNA synthesis per cell compared to controls when cultured from day 3 to day 4 but not when cultured from day 4 to day 5 of gestation.

4. I demonstrated that treatment of male rats for four weeks with 6 mg/kg/day did not shatter the paternal genome or

prevent paternal chromosomes from replicating in dividing embryonic cells. Embryos sired by cyclophosphamide-treated males clearly contained paternal chromosomes on day 4 of gestation.

5. I demonstrated that the decrease in embryonic cell number in day 4.5 embryos sired by cyclophosphamide-treated males was proportional in both inner cell mass and trophectoderm lineages, despite the selective effect on inner cell mass-derived embryonic cells in the day 7 implantation site.

6. At the electron microscopic level, I demonstrated that there were no gross morphological defects that could clearly be connected to paternal cyclophosphamide exposure in embryos examined on days 2.5, 3.0 and 4.0 of gestation. Embryos sired by treated males were compacted by day 3.0 of gestation, similar to control embryos. I showed that on day 4.0 of gestation the majority (66%) of embryos sired by treated males had formed a blastocoele cavity and undergone differentiation of inner cell mass and trophectoderm cell lineages. The remaining day 4.0 embryos (33%) sired by treated males, which had not formed a trophectoderm ring, showed signs of increased autophagy.